A Thesis for the Degree of Master of Science

Studies on Transcriptional and Epigenetic Mechanisms of *NANOG* gene in Chicken Primordial Germ Cells

닭 원시생식세포에서 NANOG 유전자의 전사 및 후생 유전 조절 기전에 대한 연구

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By

HYUN GYO JUNG

Biomodulation Major

Department of Agricultural Biotechnology

Graduate School, Seoul National University

SUMMARY

Primordial germ cells (PGCs), the precursors of sperm and oocytes, are the only cell type which can transfer genetic information to next generation. These distinct characteristics are largely dependent on germ cell-specific gene expression. They are controlled by coordinated actions of many key regulators such as transcription factors (TFs), RNA Binding proteins (RBPs) and specialized epigenetic modification.

Nanog, the mostly conserved protein between species, are well-known to be a core transcriptional factor in the early formation of embryonic development. *Nanog* is also expressed in cells from pluripotent cells to gonads through the developmental status of PGCs, which means that the role of *Nanog* is to perpetually maintain germ cell characteristics containing stemness and germness in general germ cell development. Indeed, *Nanog* regulates PGCs by signaling pathways with other key transcription factors. Furthermore, induction of mouse PGC-like cells(PGCLCs) from epiblast-like cells(ESCs) is available with *Nanog* alone. In chicken, *Nanog* expression is observed from early development. And then, the pattern of the gene is restricted to PGCs after HH3. These results suggest that *Nanog* in chicken is a key factor of regulation of PGC characteristics.

Recent studies find that epigenetic regulations, such as loss of 5mC in whole genome, DNA methylation, histone and chromatin modifications have a crucial role in PGCs. However, studies of histone and

chromatin modifications and also the control of epigenetic patterns in chicken are highly limited. In the previous studies, methylation and acetylation during germ cell specification to differentiation activate germ cell specific genes and repress somatic cell genes in mouse. And also, in chicken, germ cells are also epigenetically regulated. In contrast to mammals, the H3K27me3 global level is reduced, whereas the H3K9me3 level is increased in chicken with still acetylation level in PGCs yet unknown.

In this study, we investigated the elaborate regulatory mechanisms that govern epigenetic and transcriptional programs of *Nanog*. Histone deacetylase (HDAC) regulates *NANOG* in PGCs. And also, methylation patterns of CpG islands in c*Nanog* upstream, the regions which the transcription factors bind to, are hypomethylated in PGCs. The result of this study using a series of experiment for functionality testing, including siRNA mediated knockdown, overexpression, immunocytochemistry, luciferase reporter assay, TFs motif analysis, and western blotting demonstrates that chicken has specific epigenetic regulation during PGC development. Intriguingly, we showed that transcriptional program of NANOG was strictly regulated by specific isotype of HDACs (HDAC1 and HDAC2) and the REST repressor complex, suggesting that avian PGCs have the different molecular regulatory mechanism from that of mammals.

In conclusion, chicken PGCs display the unique epigenetic and transcriptional program of *Nanog*. Our findings first time provide valuable

insight on chicken PGCs to unravel important regulatory components as well as biological roles regarding the genetic and epigenetic regulation for stemness and germness of Nanog and better understanding of germ cell fate. The study of avian species can be adapted to important vertebrate model for the research of developmental biology and speciation.

Keywords: Chicken, Epigenetic Modification, NANOG, VASA, CoREST, Primordial Germ Cells, Transcription Factors

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LIST OF ABBEREVIATION

AP2γ : Transcription factor AP-2 gamma

BLIMP1: B lymphocyte-induced maturation protein 1

BMP4: Bone morphogenetic protein 4

CHIP: Chromatin Immunoprecipitaion

CVH: Chicken vasa homolog

DAZL: Deleted in azoospermia-like

DMEM: Dulbecco's modified eagle medium

eGFP: Enhanced green fluorescent protein

EGK: Eyal-Giladi and Kochav

ESC: Embryonic stem cell

FBS: Fetal bovine serum

FGF: Fibroblast growth factors

GAPDH: Glyceraldehyde-3-phosphate dehydrogenase

HDAC: Histone deacetyalse

KLF4: Kruppel-like factor 4

LIF: Leukemia inhibitory factor

MEF: Mouse embryonic fibroblast

OCT4: Octamer binding transcription factor 4

PGC: Primordial germ cell

PRDM14: PR domain containing 14

qRT-PCR: Quantitative reverse transcription PCR

REST: RE1 silencing transcription factor

RNAi: RNA interference

siRNA: Small interacting RNA

VPA: Valproic Acid

CHAPTER 1.

GENERAL INTRODUCTION

Primordial germ cells (PGCs), the precursors of sperm and oocytes which contains genetic information of organisms, are regulated by coordinated actions such as Transcription factors (TFs), RNA binding proteins (RBPs), and Epigenetic modifications to acquire germ cell specification, migration and the ability to differentiate into mature germ cells. Regulatory sequences in DNA region is a binding site of TFs to become an enhancer or repressor of the genes. In many cell types of vertebrates, TFs orchestrates gene expression by binding in characteristic motifs to procure adequate components of transcriptional machinery. And also, Chromatin epigenetic modifications, such as DNA methylation and histone post-translational modifications (PTMs) have a role in regulation of cell characteristic such as pluripotency and germness in PGCs (Ng, Kumar et al. 2013). Despite of the diversity of the molecular mechanisms of germ cell specification in various species, major regulators to maintain germ cell characteristics during development are well conserved (Extavour and Akam 2003). During early development in many vertebrates, the patterns of DNA methylation and the associated chromatin remodeling are the crucial steps to undergo developmental steps of PGCs.)

Nanog, a core pluripotency factor, is important for acquisition and maintenance of pluripotency during embryonic development with exclusive expression of its protein in both pluripotent cells and unipotent PGCs in mice (Chambers, Colby et al. 2003, Yamaguchi, Kimura et al.

2005), with its role in PGCs little known (Chambers, Silva et al. 2007, Yamaguchi, Kurimoto et al. 2009, Carter, Davis-Dusenbery et al. 2014). The expression pattern of mouse NANOG appears in the inner cells of the morula of blastocyst and then be expressed in the epiblast up to early post implantation stages (Hart, Hartley et al. 2004). At this time, Nanog expression appears in PGCs only after migration, and down regulated when the PGCs differentiate into adult germ cells (Yamaguchi, Kimura et al. 2005). Nanog in *in vitro* mouse model, epiblast-like cells (EpiLCs) developed from naïve pluripotent embryonic stem (ES) cells cultured in basic fibroblast growth factor (bFGF) and activin A. And also, EpiLCs acquire competence for a PGC-like fate to induce PGC-like cells (PGCLCs) with germline TFs Prdm1, Prdm14 and Tfap2c which are important for PGC specification(Murakami, Gunesdogan et al. 2016) and bone morphogenetic protein 4 (BMP4) (Chai, Yuen et al. 1999, Hayashi, Ohta et al. 2011). In chicken, *NANOG* is expressed broadly in developmental stage with germ cell restricted expression pattern of protein. Chicken *NANOG* is restrictively expressed in scattered cells over the epiblast only in what seems to have germ cell fate throughout the epiblast at stage HH1 or HH3 (Canon, Herranz et al. 2006). In result of the studies, *Nanog* is revealed as an important regulator both in pluripotent cells and PGCs. Thus, many studies have been tried to identify the transcriptional regulators to enhance or repress of *Nanog* gene to deeply understand the molecular mechanisms how cell-specific *Nanog* expression is regulated.

Recent studies find that epigenetic regulations like loss of 5mC in whole genome, DNA methylation, histone and chromatin modifications have a crucial role in PGCs. Recent studies find that epigenetic regulations like loss of 5mC in whole genome, DNA methylation, histone and chromatin modifications have a crucial role in PGCs to eliminate paternal imprinting in mammals (Kawasaki, Lee et al. 2014). However, studies of histone and chromatin modifications and also the control of epigenetic patterns in chicken are highly limited. In the previous studies, methylation and acetylation during germ cell specification to differentiation activate germ cell specific genes and repress somatic cell genes in mouse. And also, in chicken, the regulation is regulated epigenetically. In contrast to mammals, the H3K27me3 global level is reduced, whereas the H3K9me3 level is increased in chicken. However, other epigenetic modifications such as the level of acetylation/deacetylation level in PGCs still yet unknown in vertebrates.

Acetylation/deacetylation regulates gene expressions by relaxation or condensation of chromatin structure by Histone acetyltransferase (HAT) and Histone deacetylase (HDAC), respectively (Grozinger and Schreiber 2002). CoREST, NURD, and SIN3A complexes, the three major Class I HDAC complexes are abundantly existed in cells which have pluripotency. The CoREST complex in both mouse and human has been shown to establish and maintain pluripotency in the formation of

iPS cells (Yang, Wang et al. 2011), and the SIN3A/HDAC complex in mouse ESCs increase transcriptional functions with NANOG and also reprogramming efficiency. This means that HDAC can co-localize and co-activate with NANOG to efficiently regulated transcriptional programs at the chromatin level (Saunders, Huang et al. 2017). The CoREST ([co]repressor for element-1 silencing transcription factor) complex was firstly identified in mammals to be associated with the repressor for element-1 silencing transcription factor (REST)/neuronal restrictive silencing factor to have a crucial role in regulating neuronal gene expression and neuronal stem cell fate (Su, Kameoka et al. 2004). CoREST proteins are conserved chromatin modifying complexes composed of integral subunits. REST is broadly expressed in mouse early development (Chen, Paquette et al. 1998, Grimes, Nielsen et al. 2000). Mouse CoREST mRNA at E8.5 strongly expressed in head mesenchyme and becomes to have an omnipresent expression by E11.5 (Grimes, Nielsen et al. 2000). Using Knockout model of REST in mouse, REST was validated to regulate retarded cell growth and also the death of widespread apoptotic cells in E9.5 to E11.5 (Chen, Paquette et al. 1998) These complexes combine *histone demethylase and deacetylase and have been identified in* many species, such as *Drosophila (Dallman, Allopenna et al. 2004)*, *C. elegans (Smialowska and Baumeister 2006)* and mammals (Cowger, Zhao et al. 2007). CoREST complex is also associated with lincRNA HOTAIR in breast cancer (Gupta, Shah et al. 2010, Tsai, Manor et al. 2010). These results suggest that

CoREST might have a role in germ cells. NANOG also has a crucial role in embryonic and germline development, and also establish naïve pluripotency in the final reprogramming stage (Saunders, Faiola et al. 2013).

In summary, evolutionally well-conserved NANOG has many roles in pluripotency cells and also, unipotent PGCs in many vertebrates. However, TFs and Epigenetic regulation of NANOG, especially histone acetylation in the chicken PGCs are mostly unknown. Therefore, it will be valuable to investigate the molecular and epigenetic mechanisms that maintain cell characteristics in the chicken PGCs, thus providing information about the germ cell development.

CHAPTER 2.

LITERATURE REVIEW

1. Primordial Germ Cells

The first germline cell population is called primordial germ cell(PGC)s in various species (Saitou and Yamaji 2010). Primordial germ cell(PGC)s, the precursors of both the spermatogonia and oocytes, have totipotent state that undergo meiosis to generate gametes and only can deliver genetic information to next generation. Because of their unique functions, PGCs have different regulatory system from other early developmental cells, and are regulated by transcription factor, signaling pathway and epigenetic regulation. Primordial germ cells in many species have different mode of development during embryonic development.

1.1 PGC specification in germ cells

PGC development commonly undergoes early and late germ cell development – PGC specification, migration into genital ridges, and germ cell differentiation. In mammals, PGCs are specified during early germ cell development. Fertilization of the oocyte by the sperm is the first step of PGC specification. This results in a totipotent zygote that give rise to all cell lineages of an organisms including germ cell lineage itself. Thus, PGC specification is a crucial step for the acquisition of totipotency. In *C. elegans,*

D. melanogaster, zebrafish and X. laevis, germ cell specification occurs by preformation and identity of germline is continuously passed via the oocyte to the PGCs during early embryogenesis and the PGCs inherit maternally supplied germ plasm (preformation mode) Germ plasm complex is composed of RNA and conserved proteins in many species and these proteins have a role as RNA binding factors and regulate mRNA translation. Otherwise, specification of germ cell fate in mice occurs by induction and the germline must be induced from a subset of embryonic cells and are induced by cell signaling (induction mode) (Extavour and Akam 2003).

1.1.1 Preformation model

In *Drosophila melanogaster*, oocyte has localized mRNA and protein before fertilization. Polar granule is assembled in the posterior region of the oocyte (Illmensee and Mahowald 1974), and oskar, a component of polar granule, forms pole cells in posterior region and to have the maternal pore plasm proteins like vasa, tudor, valois after celluarization. (Mahowald 2001).

In *Caenorhabditis elegans*, electron dense mRNA-protein complex called P granules are scattered throughout cytoplasm in one-cell embryo (P0), and then move to the posterior cytoplasm of the embryo

during zygote formation after decision of embryonic polarity(Hird, Paulsen et al. 1996). During embryo development, P granules are firstly distributed to germline blastomere P1, and repeat division asymmetrically for 3 times so that the P4 blastomere of 16 to 24 cell stage become the PGC (Deppe, Schierenberg et al. 1978, Strome and Wood 1982, Sarmah, Muralidharan et al. 2013). The P_4 cell always becomes the PGC overall the nematodes. VASA homolog (one of the components of P granule) and Sm protein (spliceosome component) have a crucial role in the distribution of PGCs with maternal inheritance of the germ plasm (Arkov and Ramos 2010). In this species, RNA-binding proteins like VASA-related RNA helicases, the Tudor-domain proteins, NANOS, the Arg methyltransferse PRMT5 and Argonaute proteins are the conserved major germ plasm elements to determine and maintain germ cell fate (Strome and Updike 2015).

In several vertebrate species like *Xenopus laevis,* PGCs are specified by preformation mode. As in flies, germ plasm, in which RNAs, proteins and mitochondria are accumulated, is assembled during oogenesis and localized to the vegetable pole (Tada, Mochii et al. 2012). During cleavage stages, maternally loaded germ granules, segregated in vegetal plasm, finally accumulate to the PGCs has also been documented in these vertebrates. The transmission of maternal germ granules evolved independently with absence of maternal germ granules in early embryos (Chang, Torres et al. 2004).

1.1.2 Induction mode

Induction mode is conserved in most mammalian species. PGC specification to acquire germ cell fate occurs by transcriptional factors and extrinsic signals because of no maternal germ plasm in these species. Bone morphogenetic proteins (BMP) signaling pathway and WNT signaling pathways are two common pathways in human and mice (Tang, Kobayashi et al. 2016). In this signaling pathways, small set of signaling molecules and also the zygotic transcription factors critically regulate instruction of a small number of proximal epiblast cells becoming PGCs (de Sousa Lopes, Hayashi et al. 2007).

1.1.3 PGC migration in chicken

The migration of PGCs has been studied in numerous species in vertebrates, for example, mouse, zebrafish and Drosophila. In zebrafish, *Dnd1* regulates the migration and polarization of PGCs for the initial onset of germ cell migration (Weidinger, Stebler et al. 2003). In Drosophila, endoderm − 1 (Tre1) traps germ cells to regulate initiation of migration (Kunwar, Sano et al. 2008).

Especially, CXC motif receptor (CXCR4)/Stromal derived factor (SDF1) system is used to make it possible. SDF1 and its receptor, CXCR4 are also essential in both mouse and zebrafish (Ara, Nakamura et al. 2003, Molyneaux, Zinszner et al. 2003). In chicken, SDF1 and CXCR4 were determined to be expressed in the habitat where PGCs reside in after the later stages of their migration and also during migration (Stebler, Spieler et al. 2004).

Chicken primordial germ cells were first identified in the germinal crescent region and arise from epiblast and migrate from anterior of the primitive streak to the germinal ridge. In this region, PGCs find their way to the gonad using blood vessels.

In stage X (EG&K), avian PGCs or their precursors are localized in the central zone of the area pellucida on the ventral surface of the epiblast. During stage XI to XIV, these cells progressively translocate into the hypoblast. PGCs arisen from the epiblast are carried to the germinal crescent region at stage 4 to 8, and then they migrate into the genital ridges via blood stream, the circulatory system, at stage 9 to 10 (Swift 1914). PGCs have a distinct genetic differentiation mechanism in male and female embryos. In genetically female embryos, PGCs differentiate into oogonia after 8d of incubation, whereas PGCs in genetically male embryos differentiate into spermatogonia after 13d of incubation.

The unique migration patterns of PGCs through the circulatory system are the characteristics of avian species and these characteristics can be used for the tools for the study of early PGC development, a laborious technique in other species.

1.2 Signaling pathways of PGCs

Based on the several researches of maintenance of PGCs *in vitro*, such as BMP4's function of activation of germ cell specific genes, *Prdm1* and *Prdm14* (Ohinata, Ohta et al. 2009), extrinsic factors to stimulate signal transduction have crucial roles in PGC development as the key transcriptional regulators (Saitou and Yamaji 2010).

The growth factors, BMP4, LIF, SCF, retinoic acid and FGF, which are contained in each signal, are required for PGC survival and proliferation (Whyte, Glover et al. 2015). In mammal, using a cocktail of growth factors, KLF4, LIF, BMP4, SDF-1, bFGF and compounds (N-acetyl-l-cysteine, forskolin, retinoic acid) enable the survival and self-renewal of PGCs in the absence of somatic cells in culture (Farini, Scaldaferri et al. 2005).

Leukemia inhibitory factor (LIF), basic fibroblast growth factor (bFGF), bone morphogenetic protein 4 (BMP4) and kit ligand are the well-known signaling pathways of primordial germ cells related to proliferation and survival of primordial germ cells (De Felici and Barrios 2013).

Like-wise other species, chicken PGCs also proliferate and the proliferation of PGC during embryogenesis, specification, differentiation and also PGC survival is controlled by a cell signal.

1.2.1 Transforming Growth Factor-Beta (TGF-β) signaling pathway

Transforming Growth Factor-Beta (TGF-β) super family is composed of a large and diverse polypeptide morphogen groups: TGF -β themselves, Bone Morphogenetic Proteins (BMPs) and the Growth and Differentiation Factors (GDFs) (Ripamonti, Ferretti et al. 2009). TGF-β is activated from a complex before binding to its receptors. Ligand binding to receptors by highly conserved juxta-membrane region, known as the GS domain. TGF-β family members have distinct temporal and tissue specific expression patterns so that they play crucial roles in the development and also repair of tissues in organisms like immune cell lineages, dendritic cells, etc (Yan, Liu et al. 2009)

In the result of screening signaling pathway and genes related to male PGC production, TGF-β is related to the regulation of male germ cell differentiation from chicken ESCs. During induction to PGCs, Expression of Nanog and Sox2 in ESCs are decreased. In contrast, Stra8, Dazl, Integrin-α6 and c-kit, the germ cell specific genes, are increased. In the result of study, TGF-β regulates germ cell differentiation (Zhang, Wang et al. 2016).

1.2.2 Bone morphogenetic protein (BMP) signaling pathway

In mice, PGCs require Bmp2 from the visceral endoderm and Bmp4 and Bmp8b from extra-embryonic ectoderm. Expression of *Bmp4* and *Bmp8*b from the extra-embryonic ectoderm for generation of sufficient cell numbers to induct for germ cell (Chai, Yuen et al. 1999, Ying, Liu et al. 2000, Ying and Zhao 2001). Also, key transcriptional regulators Blimp1 (Prdm1), Prdm14 and AP2γ in competent epiblast cells express to respond BMP signals to have a crucial role in PGC specification. A tripartite transcription factor network represses somatic mesodermal program, activates germ cell genes, and also resets epigenome as a basal state. (Magnusdottir, Dietmann et al. 2013).

In human, SOX17 from endoderm is the key regulator of hPGCLCs acting in the upstream of Blimp1 to promote germ cell development (Irie, Weinberger et al. 2015). In contrast, only the proximal epiblast cells receiving BMP signaling can induce PGCs expressing Blimp1 from somatic tissues (Kanai-Azuma, Kanai et al. 2002).

1.2.3 Stem cell factor (SCF) Signaling Pathway

The ligand stem cell factor (SCF), and its receptor c-kit, are essential for PGC survival and migration in the mouse (Matsui, Zsebo et al. 1990, Dolci, Williams et al. 1991, Gu, Runyan et al. 2009), and also, this signaling pathway, which is important for germ cell survival and migration in mice has also been identified in the chicken. Chicken c-KIT is expressed in both stage 19HH embryos in the presumptive genital ridge and cultured chicken PGCs (Tang and Zhang 2007). Similar to many other genes, the in *ovo* studies of SCF in avian germ cells has not been determined yet. SCF without addition of hSCF or mSCF for PGC culture derivation has been used in cultured PGC medium (van de Lavoir, Diamond et al. 2006, Macdonald, Glover et al. 2010, Rengaraj, Zheng et al. 2010). SCF is requires as an PI3K inhibitor, a down-stream target of c-kit. This results in a loss of PGC proliferation in culture (Macdonald, Glover et al. 2010).

1.2.4 Basic fibroblast growth factor (bFGF) signaling pathway

Basic fibroblast growth factor (bFGF) ligand activates the Map kinase signaling pathway. Mitogen – Activated Protein Kinases (MAPKs) are activated by many different cell signals. MAPKs deliver extracellular signals from activated receptors. Especially, in nucleus, MAPKs regulates appropriate gene regulatory by activation of gene transcription, protein synthesis, cell cycle regulation, apoptosis and differentiation. bFGF signaling in PGCs inhibition of mitogen – activated protein kinase (MEK) results in reducing of proliferation of cultured PGCs (Macdonald, Glover et al. 2010, Rengaraj, Zheng et al. 2010).

1.3 Germness related genes in Chicken

Specification and maintenance of PGC characteristics are determined by major factors and germ line specific RNA-binding proteins such as VASA, DAZL and DND1. These proteins may have a role in repressing translation to prevent their differentiation into somatic cells and to maintain PGC characteristics (Houston and King 2000).

The conserved and universal molecular determinants in germ line maintenance are called *vasa* and *vasa*-like DEAD box RNA helicase genes. Vasa is a member of the DEAD box protein family contains nine conserved sequence motifs to unwind duplex RNA in a discontinuous and manner to have a role in pre-mRNA splicing, ribosome biogenesis, nuclear export, translational regulation and degradation. The *vasa* gene firstly isolated in *Drosophila* (Lasko and Ashburner 1988), and *vasa* homologous genes have been characterized in germ cells across diverse organisms. In some organisms like mouse, *vasa* only found in late germ cell development stage because of no maternal inheritance in germ plasm (Toyooka, Tsunekawa et al. 2000).

In chicken, PGC specification is yet unknown problem. However, studies of germness genes are continuously studied in views of their characteristics and expressions. Chicken *vasa* homologue (*CVH*) is one of the germ plasm components detected in initial developmental stages and is also localized to cytoplasm of germ cells and co-localized to spherical mitochondrial cloud in growing oocytes (Gustafson and Wessel 2010). Early developmental expression of *CVH* in chicken embryos supports that chicken PGC specification may follow the predetermination model determined by maternally inherited factors and is restricted to chicken germ cells during early embryogenesis (Tsunekawa, Naito et al. 2000). Tracking cells using Cvh promoter - GFP expression vector shows the clue of Cvh expression's crucial role in differentiation into the germ cell fate and germline competency. These result also suggest that the usage of Cvh as a marker of

germline competency in newly derived pluripotent cells (Lavial, Acloque et al. 2009).

Dazl (Deleted in Azoospermia-Like) is a member of the *DAZ* gene family. Dazl regulates translation in various organisms as a germ cell specific RNA-binding protein (Collier, Gorgoni et al. 2005). *DAZL* regulates spermatogenesis and oogenesis (Ruggiu, Speed et al. 1997). *Dazl* gene also protect germ cells in the from apoptosis (Lin and Page 2005). In chicken, *DAZL* is detected in germ cells until the adult germ cell (Rengaraj, Zheng et al. 2010), and *cDAZL* (chicken *DAZL*) is expressed specifically in PGCs during their migration from EGK stage X to HH stage11. This is the difference from mammalian species. In mammalian species, *DAZL* is expressed in late PGCs and regulate pre-meiotic and meiotic genes to make fertile germ cells in both sexes, for example, fetal gonocytes, spermatogonia and spermatocytes in adult testis (Fu, Cheng et al. 2015). Therefore, study of *cDAZL* expression pattern suggests origin and central formation of PGCs in chicken and the difference between other species (Lee, Choi et al. 2016). Above experiments indicate that germ cell-specific RNA-binding proteins are required for germ cell development and survival.

2. NANOG

Nanog is a multi-domain protein of which contains two transactivation domains, N-terminal transactivation domain (ND) and C-terminal transactivation domain (CD) and Tryptophan-rich domain (WR) (Saunders, Faiola et al. 2013).

To understand the specific gene expression, confirmation of gene expression induced by deletion or mutation of targeting site predicted by specific gene promoter analysis. In the research of Nanog promoter, there are identification in mice and human. In mice, 180bp before transcription start site (TSS), and, in human, 118bp before TSS has OCT4/SOX2 binding site and has a crucial role of Nanog gene expression (Kuroda, Tada et al. 2005, Rodda, Chew et al. 2005). This region is highly-conserved between species. Nanog promoter in mice is activated by binding with protein factors; STAT3, T and FOXD3, and is repressed by factors like p53 and TCF3 (Pan and Thomson 2007). And also, OCT4 and SOX2 interact with KLF4 and PBX1 protein to regulate Nanog in human ESCs (Chan, Zhang et al. 2009).

2.1 NANOG in PGCs

Induction of PGCs is regulated by transcription factors and epigenetic changes to upregulate its characteristics. In the previous researches, there were few factors to have a key role in this mechanism.

Nanog, a key pluripotency factor in Inner Cell Mass (ICM) of Blastocyst, also expresses in PGCs (Yamaguchi, Kimura et al. 2005). Epiblast-like cell(EpiLC)s are induced from naïve state embryonic stem cell(ESC)s by Nanog with basic fibroblast growth factor (bFGF) and activin A in vitro and acquire PGC-like characteristics (Hayashi, Ohta et al. 2011). In recent study, in contrast to the result that bone morphogenetic protein 4 (BMP4), PR domain zinc finger protein 1 (Prdm1; Also known as Blimp1), PR domain zinc finger protein 14 (Prdm14) and Transcription factor AP-2 gamma (Tfap2c) are necessary to the induction of Primordial germ cell-like cell(PGCLC)s from EpiLCs, Nanog can induce PGCLCs by induction of established 4 PGC specific transcription factors (Murakami, Gunesdogan et al. 2016).

2.2 NANOG in early development

Nanog protein is a transcription factor that binds to consensus sequence ((C/G)(G/A)(C/G)C(G/C)ATTAN(G/C)) (Mitsui, Tokuzawa et al. 2003). Nanog have some roles in different states. Nanog interacts with the main self-renewal factors, Oct4, Sox2, Rex-1 synergistically and cyclically to contribute to self-renewal (Shi, Wang et al. 2006, Schulz and Hoffmann 2007). And also, Nanog binds to the promoter of Gata6 and represses Gata6 gene expression (Singh, Hamazaki et al. 2007). Nanog directly binds to NF-

kB, so that Nanog represses transcriptional activation of NF-kB, the gene induces differentiation, and maintains the characteristics of stem cells with Stat3 (Torres and Watt 2008).

2.3 NANOG in Medaka fish

In *Medaka fish*, Nanog has a different regulation with mammal and it is required for proliferation and S-phase transition in embryonic development. Thus, zNanog, which has a homology with mammalian Nanog, generates protein in nucleus and express mRNA in cytoplasm to repress PGCs' proliferation in embryonic development (Wang, Liu et al. 2016).

2.4 NANOG in Chicken

In chicken, the pattern of Nanog is differ from mice. Nanog in chicken is not shown in hypoblast in HH1 to HH3, and is shown as scattering shape in cells in which have a fate to become PGCs in the germinal crescent between Area pellucida and Area opaca at HH5 to HH8. After that, Nanog expression cells have same patterns with chicken PGC

specific genes, Vasa and Dead. Continuously, Nanog is also expressed in PGCs from HH12 to HH17 – HH20, when PGCs migrate into gonad (Canon, Herranz et al. 2006).

2.5 Epigenetic regulation of NANOG

In the epigenetic side, Nanog has two main roles: methylation and chromatin condensation. In methylation, Nanog has an interaction with Prdm14 correlated to PRC2, H3K27me complex, to regulate genes like fibroblast growth factor receptor 1/2 (Fgfr1/2), which determine system. This results in repression of differentiation. And also, it helps broad hypo-methylation using De novo DNA methyltransferase, Dnmt3A/3B/3L (Nakaki and Saitou 2014). Activators of Key enhancers marked by H3K4me1/2, and these also marked by H3K27ac. MLL4/KMT2D is a main enhancer to p300, which is an acyltransferase and is required for H3K27ac. Nanog has an interaction with MLL4 and this results in relation of Nanog and H3K27ac (Wang, Lee et al. 2016).

In chicken, Nanog expression has a role in PGC proliferation and survival similar to mammals and is observed in HH12 to HH17-HH20, and this means Nanog regulates PGC migration from vascular system to genital ridge (Canon, Herranz et al. 2006). Methylation pattern of Nanog promoter

is hypo-methylated in sperm and strongly hyper-methylated in other somatic cells. This pattern is also same in Ddx4, Dnd1 and Dazl, which are related to chicken PGC proliferation and survival with Transcription factors unknown (TANAKA, Tomoki et al. 2014).

In Chromatin level, Nanog also have some different roles. Formation of opened and uncondensed chromatin defines the quantity of pluripotency. Nanog in mouse ESCs regulates pluripotency related to the formation of heterochromatin. In time, Sall1, heterochromatin related protein, maintains opened chromatin state with low level of H3K9me3 by direct co-factor (Novo, Tang et al. 2016).

Nucleosomes are basic units of chromatin that which is the histone compaction of DNAs. Nucleosomes are known to have a crucial role in packing of genes. Nucleosome itself works as an epigenetic regulator with the most crucial transcription factor, Oct4, competitively. This regulation forms nucleosome depleted region (NDR) in DNA regulatory region of Nanog to start the transcription, and nucleosome forms NDR before the regulation of Oct4 to regulate de novo DNA methylation (Hammachi, Morrison et al. 2012). As the result, Nanog has a possibility to have interactions with other transcription factors to regulate DNA and histone modification in chicken PGCs.

2.6 Recent study of NANOG

In recent studies, Nanog－RNA binding proteins, YBX1 and ILF3 upregulate pluripotency related genes in mouse ESCs (Guo, Xue et al. 2016). Prmt7 regulates micro RNA, miR 24-2, which represses Oct4, Nanog, Klf4 and c-Myc, to regulate stem-ness (Lee, Chen et al. 2016). And also, Cbfa2t2 combine with mouse Prdm14 and Oct4 to increase pluripotency related genes and lineage-specific genes. This gene also increases repressed chromatin modification genes to regulate PGC expression from ESCs (Tu, Narendra et al. 2016). Induction of germ cells also are related to *Nanog* gene, known as a core pluripotency factor. *NANOG* can induces germ cells in primed epiblast *in vitro* by interacting enhancers of *Prdm1* and *Prdm14* in EpiLCs in mouse (Murakami, Gunesdogan et al. 2016).

3. Transcriptional gene regulation

Gene expression regulated by Promoter is spatially and temporally controlled. Transcriptional regulation by promoter results in signaling pathways and this transcriptional regulation is managed by the interactions between transcriptional factors including enhancers, silencers, insulators in cis-regulatory region of promoters (Novina and Roy 1996, Goodrich and Tjian 2010). Specific gene expressions are the result of one or more

transcriptional factors on promoters called enhancers and repressors. Thus, studies on *cis* elements and different algorithms of specific gene promoters and also transcriptional start points and transcription factor binding sites is important for exploring the modulation of gene expression regulatory network. (Bucher, Fickett et al. 1996, Fickett and Hatzigeorgiou 1997).

3.1 Promoter

Promoter is a direct tool of gene regulation and exists in the upstream of gene. In the upstream of gene, mRNA synthesis in eukaryotic cells initiates by complex and highly regulated process. This process is the result of general transcription factors and RNA polymerase II which assemble into a pre-initiation complex at the binding motif of the core promoter. (Struhl 1989, Da Silva and Srikrishnan 2012).

Regulatory elements that map near genes are *cis*-acting DNA sequences. Cis-acting DNA sequences contains two elements: Core promoter and Promoter Proximal Elements. Core promoter has a basal level expression which contains binding site for TATA-binding protein and associated factors. Promoter Proximal Elements has a level for transcriptional regulation and also translational regulation. These elements contain binding sites for transcription factors. Eukaryotic Promoter elements

are so various that their combinations of core and proximal elements are found near each gene.

3.1.1 Promoter Proximal elements

Promoter proximal elements has key roles in gene expression in many developmental stages and also in many different type of cells, surprisingly, also in ubiquitously expressed genes. Activators which are important in transcription regulation are recognized by promoter proximal elements.

Housekeeping genes such as β-actin are ubiquitous in all cell types for basic cellular functions. These genes have common promoter proximal elements because of their ubiquitous characteristics. Also, housekeeping genes are recognized by activator proteins found in all cells. Otherwise, Genes expressed only in some specific cell types or at singular times have promoter proximal elements recognized by activator proteins found only in specific types or times.

3.1.2 Core promoter

The definition of core promoter is as the minimal DNA region sufficient to direct low levels of activator-independent (basal) transcription by RNAP II in vitro (Butler and Kadonaga 2002). Core promoter typically extends approximately 40bp up-and down- stream relative to the transcription start site and can contain several distinct core promoter sequence elements recognized by the general transcription factor TFIID which promotes the process of forming pre-initiation complexes (Goodrich and Tjian 2010).

Core promoters in higher eukaryotes are highly diverse in structure, and, so far, only TATA box which contain an A/T-rich sequence, with the consensus TATAAA, and initiator element are shown to be capable of directing accurate RNAP II transcription initiation independent of other core promoter elements. TATA-mediated transcription initiation is well known. Nevertheless, initiator and other core promoter elements are known very little(Kadonaga 2012).

In the past, the core promoter was regarded as being found in every cellular gene. Nowadays however, not only distal promoters and enhancers but also core promoter and initiators are involved in transcriptional regulation by interacting cell-specific trans-acting factors and mechanisms(Juven-Gershon and Kadonaga 2010). Also, germ cells are observed to express core promoter-associated regulatory factors with various core promoter architecture contribute to regulate germ-cell-specific

transcriptional signals of appropriate gene expression related to somatic gene repression and germ-ness upregulation genes(Lenhard, Sandelin et al. 2012). These studies can be adapted to germ cell transcription by specialized core promoters in application like embryonic study and production of transgenic animal using germ cell-specific promoter vectors (Bhullar, Schmidt et al. 2001, Song, Lai et al. 2016).

3.1.3 Germ cell specific promoter

Germ cell specific promoter is a unique set of core promoter-associated transcription factors not found in somatic cells. These promoters can used in producing transgenic animals including small size (~100bp) and relatively high GC content (Xiao, Kim et al. 2006).

Cell-type-specific Transcription activation factors (TAFs) and TBF related factors (TRFs) have unique roles during development, differentiation and cell proliferation. Proliferation and differentiation regulated by specific factors and promoter such as positive and negative cell cycle regulators, differentiation promoting transcription factors and epigenetic regulation, for example, chromatin remodeling promoting proliferation and differentiation (Ruijtenberg and van den Heuvel 2016).

Recent studies showed an analysis of endogenous genes and regulators observed in the context of nearly homogeneous population of single, specific, differentiated cell type and in distinct cell cycle stages. 'Non-

prototypic' core promoter recognition factors, including cell-type specific TAFs and TRFs have a critical role in driving cell specific programs of transcription to regulate specific sets of genes such as germ cell specification or maintenance of gene expression states during embryonic development (Goodrich and Tjian 2010). By using GFP reporters contain ALF promoter, which has a regulatory role in male and female gametogenesis, CCCTC-binding factor (CTCF) binds to ALF promoter to regulate germ cell gene expression. (Kim, Li et al. 2006). By research germ cell specific promoters, on-off germ cell gene promoter regulation has an insight in application for transgenic animal and developmental studies.

3.2 Enhancer

Eukaryotic enhancers are one of the *cis*-regulatory elements. Enhancers are required for maximal transcription of genes involved in tissue-specific gene expression. Enhancers can exist upstream or downstream of the transcription initiation site. Enhancers are DNA sequences that could modulate from a far distance base pairs away from the initiation site. Enhancers could regulate transcriptional ability by interaction with transcription factors independent of their location, distance or orientation. (Banerji, Rusconi et al. 1981). Ability of enhancer by interaction with transcription factors independent of their location, distance or orientation is the result of DNA looping that brings transcription factors

together (Matharu and Ahituv 2015). The distinct chromatin feature of enhancers to regulate the cell-specific gene expression mechanisms. OCT4 expression in mouse is regulated by stage/tissue specific enhancer in mouse that proximal and distal enhancer are respectively active in the epiblast of mouse embryos and in the germline cells. (Yeom, Fuhrmann et al. 1996).

3.3 Transcription factors

Transcription factors are also called activator proteins and silencer proteins by their role in gene expression. These bind to promoter, enhancer and silencer DNA by interactions with other proteins. These interactions activate/repress and increase/decrease transcription as much as 100-fold above basal levels. Transcriptional activators bind to specific promoters and enhancers at specific times to increase transcriptional levels by two structural domains: DNA-binding domain and Transcription-activator domain.

Transcription factors regulates central mechanism of promoter regulation by site-specific binding. Germ cell specific factors have their roles in germ cell specific gene regulation in many species. The gene expression program which specialized transcription in male germ cells of *Drosophila* and Mouse Primary Spermatocytes were discovered. A Testis-Specific DREAM Complex (tMAC) regulated meiotic arrest of *Drosophila* and

Cyclic AMP response element (CRE) binding protein (CREB) and cyclic AMP response element modulator (CREM) have regulatory pathway with ACT and KIF17 in haploid cells. And also, Two TBP – related factors, TRF2 and TRF3, maintains self-renewal of spermatogonia in male and restrictedly expresses in the ovary, respectively (White-Cooper and Davidson 2011).

4. Epigenetic modifications

Epigenetics including remodeling of nucleosomes, DNA methylation, posttranslational modifications of histone proteins, and chromatin reorganization modulates chromatin structure to regulate gene expression allows cell specific characteristics. Integration of TSS location with related functions, such as histone methylation (Yan and Boyd 2006) and acetylation sites (Pokholok, Harbison et al. 2005), the position of nucleosomes (Wiren, Silverstein et al. 2005), and the position of transcription factor binding sites are the clues of transcriptional regulation of proximal to genes.

Chromatin has two broad classes: Euchromatin and Heterochromatin. Euchromatin is the majority chromatin in its de-condensed state during interphase, and it only condenses during mitosis This contains most genes and this region is an active region. Heterochromatin is

highly condensed even in interphase. When the chromosome packaged, this influences gene activity. Nucleosomes in the de-compacted area unwind to make transcription initiate. Transcription factors are non-histone proteins which unwind nucleosomes and remove histones at 5' end of genes and open to interaction with RNA polymerase so that recognize promoter and initiate gene expression (Butler and Kadonaga 2002). In the result of Epigenetic regulation, DNA are modified in both DNA and Histone level (Rothbart and Strahl 2014).

4.1 Epigenetic modifications in PGCs

Epigenetics must be reset during germ cell specification and establish new epigenetic states for renewal of acquirement of totipotency (Smallwood and Kelsey 2012). Therefore, epigenetic modifiers have key roles in germ cell development in germ cell specific fate such as meiosis and maintaining genomic integrity through stage and cell specific gene regulation. In the researches of germ cell studies, germ cells acquire unique cell types by a series of epigenetic events (Sasaki and Matsui 2008).

Epigenetic regulation of germ cell development has an extensive spectrum. Epigenetic reprogramming has a crucial role in PGC mechanism to reacquire totipotency in pre-migratory and pluripotency in migratory

germ cells (Seki, Hayashi et al. 2005). It occurs from PGC specification to colonization and migration into developmental gonads of the early germ cell development, so that germ cells mature to acquire the capacity of germ cell characteristics after fertilization (Jang, Seo et al. 2013).

Epigenetic Modification has a critical role to regulate germ cell specific gene and establish the characteristics of PGCs. During migration of PGCs into gonads, DNA methylation and H3K9me2 is decreased, and H3K27me3 is increased. After migration into gonads, H3K27me3 is hypo-methylated to regulated germ cell specific genes (Tollervey and Lunyak 2012). Due to the features that PGCs have both activation and repression mechanism, H3K4me3 and H3K9Ac is temporarily increased during PGC development. For these reasons, Epigenetic regulation is highly important for PGCs to acquire totipotency during embryonic development. (Khromov, Pantakani et al. 2011).

4.1.1 Epigenetic modifications in Chicken PGCs

In Chicken, transcriptome analysis of primordial germ cell – specific genes including genes related to cell cycle was reported, and also the result that chicken's embryonic stem cell is similar to mouse's primordial germ cell was reported (Rengaraj, Lee et al. 2012, Jean, Oliveira et al. 2015).

Nevertheless, some of the germ-cell specific regulator genes are differently expressed differ from mouse.

In case of the genes, SOX2/SOX3, SOX2 expresses mostly in cultured cESCs. In opposition to SOX2, SOX3 expresses in cES, and also PGCs and Hypoblast (Zhang and Klymkowsky 2007, Acloque, Ocana et al. 2011). DAZL, DND1, DDX4, PIWIL1 and GTSF1, CALR3, GPR149 is involved in differentiation of mammalian germ cells, (Edson, Lin et al. 2010, Ikawa, Tokuhiro et al. 2011, Jean, Oliveira et al. 2015) and they also have a role of germ cell specific genes in avian species (Lee, Choi et al. 2016, Sekinaka, Hayashi et al. 2016). In spite of the fact that OCT4/POUV, NANOG, ENS/ERNI, CDX2's expression in both cESCs and PGCs, TRIM71, KLF1, SOX3, CFC1B, OTX2 and EOMES expresses only in cESCs, and KLF2, DAZL, DDX4 express only in PGCs.

Epigenetic regulation of chicken PGCs was not unknown until recently. In Chicken, Upregulation of the gene, which transcribes SUV39H2/KMT1B, Histone Methyltransferase, results in elevation of H3K9me3, the most heterochromatin model, in cPGCs related to cESCs. In contrast, the expression of H3K27me3 is lower in chicken's PGCs. DNA methylation in PGCs regulates the expression of DNMT1, DNMT3B and HELLS/SMARCA6 to maintain its pattern and have an important role. In result, epigenetic regulation of chicken PGCs differs from mouse PGCs in

that H3K9me3-abundant heterochromatin and limitative accumulation of H3K27me3 (Zhang, Elsayed et al. 2015, Kress, Montillet et al. 2016).

4.2 DNA Methylation

DNA methylation regulates gene expression usually by inhibition of transcriptions. In vertebrates and plants, many genes contain CpG islands near their promoters (Lister, Mukamel et al. 2013). CpG islands are DNA sequences in which contains lots of CG repeats (Gardiner-Garden and Frommer 1987). CpG has different pattern in housekeeping genes and tissue-specific genes. In housekeeping genes, The CpG islands are un-methylated (Schug, Schuller et al. 2005). Otherwise, Methylation pattern of tissue - specific genes are methylated in CpG islands. Transcriptional silencing via methylation blocks transcription factor binding to induce heterochromatin. Only a fraction of CpG associated promoters have TATA-like elements. Control of germ cell-specific promoters are broadly similar in several sequences like a TTCAAA element, a GC-rich region with a number of CpG island and un upstream TC-rich region (Han, Xie et al. 2004). This result means that germ cell - specific promoters could be regulated by epigenetic regulation including DNA methylation. Though the sequences are broadly similar, Factors related to their regulation has

unknown selective activation during germ cell differentiation and somatic silencing using epigenetic regulation.

4.2.1 DNA methylation in PGCs

DNA methylation leads to proper epigenetic programming of PGCs from pre-implantation development (Durcova-Hills, Hajkova et al. 2006). During germ cell development, nearly complete demethylation and re-methylation (Seisenberger, Peat et al. 2013).

DNA methylation contributed by the paternal gamete is de-methylated through a hydroxyl-methylated intermediate catalyzed by the Tet Methyl-cytosine Dioxygenase 3 (TET3) member, one of the TET family (Gu, Guo et al. 2011, Wossidlo, Nakamura et al. 2011, Smith, Chan et al. 2012). After fertilization, loss of global DNA methylation levels continues until the blastocyst stage (Guibert, Forne et al. 2012) After specification of the ICM, global re-methylation of the genome occurs (Borgel, Guibert et al. 2010). Global resetting of DNA methylation patterns occurs at germ cell development and at early in embryogenesis (Sasaki and Matsui 2008).

Generally accurate heritability of DNA methylation and its stability makes erasure of DNA methylation might only be possible either by breaking DNA or replicating DNA in the absence of DNMT1 and such

enzymes like DNMT3A, DNMT3B and also 5hmC (Reik 2007, Kangaspeska, Stride et al. 2008, Stadler, Murr et al. 2011)

During implantation of the blastocyst, the first wave of epigenetic reprogramming occurs. Existed DNA methylation patterns at CpG islands are erased to become a barrier of undergoing somatic cell fate. Only some regions related to evolutionarily young and potentially hazardous retrotransposons remain highly methylated (Tang, Dietmann et al. 2015).

When PGCs migrate to the genital ridge epigenetic remodeling observed only in paternal or maternal imprinted loci of germ cells has started. Transcriptional repression and DNA methylation decreases in migrating PGCs are regulated by H3K9 di-methylation (H3K9me2). Subsequently, proper repressive chromatin state of the PGC genome is maintained by the H3K27 tri-methylation (H3K27me3), another repressive mark. Theses repressions by methylation marker is related to repression of somatic genes and contribution of acquiring totipotency in migrating PGCs (Sasaki and Matsui 2008).

After germ cell differentiation, distinct sex-specific DNA re-methylation patterns of mature oocytes and sperm during germ cell development occur. (Durcova-Hills, Hajkova et al. 2006).

4.2 Histone Acetylation

The N-terminal tails of the histone H3 and H4 contain many Lysine residues, and can be modified by acetylation, methylation, phosphorylation, ubiquitination and SUMOylation to regulate the interactions both between histone-DNA and histone-histone (Ridsdale, Hendzel et al. 1990, Wang, Zang et al. 2009), and these post-translational modifications regulate gene transcription.

Acetylation and deacetylation of lysine residues on histone tails are related to initiation and elongation. In transcription start sites (TSSs), Chromatin remodeling in promoter region is regulated by acetylation of nucleosomes. And also, Acetylation destabilizes nucleosome structure to facilitate RNA polymerase II binding to activate mRNA synthesis result in transcription activation (Wang, Zang et al. 2009). Histone acetylation is commonly associated with transcriptional activation to plays crucial roles in modulating chromatin structure and functions (Shahbazian and Grunstein 2007). The acetylation state of a chromatin locus is controlled by two classes of antagonizing histone modifying enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs). These enzymes add to or remove from acetyl groups of target histones, respectively.

4.3 HDACs

Histon deacetylation is associated with transcriptional corepressors (Kadosh and Struhl 1997). These enzymes are highly conserved from yeast to human. The 18 HDACs in humans are grouped into four classes based on their homology with yeast orthologs and other phylogenetic analyses: class I (HDAC1,2,3, and 8), class II (HDAC4,5,6,7,9 and 10), class III (Sirt1,2,3,4,5,6, and 7) and class IV (HDAC 11) (de Ruijter, van Gennip et al. 2003, Yang and Gregoire 2005, Yang and Seto 2008). Among the four classes, HDAC1 and HDAC2 in class I mostly regulate gene expression in nucleus and the others regulate in both nucleus and cytoplasm according to the mechanism of the cells. The functions of these enzymes are various and substrate specificity between these are little. Though these enzymes have small substrate specificity, these enzymes can have a various function by their co-factors (Shi and Whetstine 2007). Among these, HDAC 1,2,3 and 8 in class I mostly regulate gene expressions using co-repressor complexes: Sin3, NuRD (nucleosome remodeling and deacetylation), CoREST (co-repressor for element-1-silencing transcription factor) and SMRT (silencing mediator of retinoid and thyroid receptors)/NCoR (nuclear receptor co-repressor) (Kelly and Cowley 2013). HDAC1 and HDAC2 are the main components of all four complexes. The most well-known HDAC1/2 complexes are Sin3A, NuRD and CoREST complex. These complexes use transcription factor and histone recognition site in complexes to have roles

in repression of genes, regulation of cell cycle and DNA repair, etc. according to the cell types.

HDAC mostly have researched in the study of cancer regulatory mechanism than embryology. For example, AML1 – ETO fusion protein, discovered in t(8;21) AML patients, represses the regulation of AML1 targeting genes attracting HDAC1/2/3 by ETO elements. And, this protein result in inhibition of bone marrow differentiation and induction of cell transformation. In the mechanism of the protein, HDAC1/2/3, DNMT1, DNMT3A and EZH2, which also regulate in mechanism of stem cells and PGCs are contained and are being researched (Gong, Li et al. 2016). And also, well-known HDAC inhibitors being used in the researches have various activities and combination ability in inhibition of specific HDACs and their complexes. For example, Romidepsin and VPA, which inhibit HDAC1/2 and broad HDACs respectively, have the highest combination ability with CoREST complexes (Robertson, Hurley et al. 2013). In mice, HDACs also repress p21 and p57 to regulate G1-to-S-phase transition with no negative effect in B-cell to improve cell proliferation (Yamaguchi, Cubizolles et al. 2010).

In terms of embryological study of HDACs, most studies in mice are divided into two developmental stages; early embryo before fertilization to Blastocyst and oocyte, the functional germ cell after migration into genital ridge of PGCs. In mice oocyte, HDACs regulate acetylation in meiosis (Kim,

Liu et al. 2003), and HDAC2 regulates de novo methylation of DNMT3A2 in oocyte, late of PGC development, to forms imprints and repress repetitive element result in protecting genomic integrity (Ma, de Waal et al. 2015). In case of HDAC1, HDAC1 repression in ESCs regulates HDAC1/2 complex to acquire specification of differentiated cell by regulating H3K56Ac (Dovey, Foster et al. 2010). In the development of mice in pre-implantation stage, SIN3A, one of the HDAC1/2 complexes, maternally regulates gene expression related to reprogramming until middle of 2-cell stage (Jimenez, Melo et al. 2015).

4.3.1 HDACs in chicken

In chicken, according to Pubmed, HDAC1,2,3,8 and 9 has Phylogenetic similarity between other species between HDACs, which translate proteins. In comparison, chicken's HDAC 1,2 and 3 have 480,488 and 428 amino acids and resemble 93.8, 97.1 and 97.0%, respectively (Takami, Kikuchi et al. 1999).

In the embryonic study of HDACs in chicken, HDAC3, Dnmt3a and Dnmt3b are highly expressed in ESCs. On the other hand, the expressions of Dnmt1, HAT and POUV are decreased to regulate the differentiation to embryonic body (EB) cells. At that time, methylation

increases and H3 acetylation decreases, so that they regulate consistent gene expression of POUV to maintain pluripotency and prevent differentiation (Jiao, Wang et al. 2013). In the result of co-treatment of trichostatin A (TSA), HDAC inhibitor, and 5-azadC, DNMT inhibitor to regulate transcription of genes, they synergistically upregulate NANOG expression and decrease overall methylation in chicken ESCs (Wang, Wang et al. 2016). And also, treatment of tamibarotene (AM80), RAR α activator, and TSA promotes STRA8 gene's promoter in chicken ESCs to induce spermatogonial stem cell (SSC)s which express Integrin β 1 (Zhang, Zuo et al. 2015).

CHAPTER 3.
Transcription factor confers germ cell specific transcriptional control of the chicken *NANOG* through CpG methylation of a proximal promoter core region

1. Introduction

Primordial germ cells (PGCs) are the precursors of differentiated germ cells, sperm and egg, has two main distinguishable properties: stemness to maintain pluripotency and self-renewal, germness to undergo PGC development from PGC specification to mature germ cell differentiation to be able to deliver genetic information to next generations. Despite of the diversity of the molecular mechanisms of germ cell specification in various species, major regulators to maintain germ cell characteristics during development are well conserved (Extavour and Akam 2003).

For examples, germ cell related genes, Vasa and Dazl , are expressed in germ line cells in various species to support the germ cell development. (Takeda, Mishima et al. 2009, Gustafson and Wessel 2010, Lesch and Page 2012). Pluripotency genes such as Nanog, Oct4 and Sox2 are also express in PGCs in many organisms. The importance of these genes in PGCs are revealed by the studies that the mis-regulation of these genes cause apoptosis in PGCs, still their functions yet unknown (Saito, Takeda et al. 2003, Kehler, Tolkunova et al. 2004, Chambers, Silva et al. 2007). The recent studies of pluripotency factor for the control of epigenetic status in germ cells assume the relationship between germ cells and the roles of pluripotency n the cells. (Leitch and Smith 2013).

Nanog, a core pluripotency factor, is important for acquisition and maintenance of pluripotency during embryonic development with

exclusive expression of its protein in both pluripotent cells and unipotent PGCs in mice (Chambers, Colby et al. 2003, Yamaguchi, Kimura et al. 2005), with its role in PGCs little known (Chambers, Silva et al. 2007, Yamaguchi, Kurimoto et al. 2009, Carter, Davis-Dusenbery et al. 2014). The expression pattern of mouse NANOG appears in the inner cells of the morula of blastocyst and then be expressed in the epiblast up to early post implantation stages (Hart, Hartley et al. 2004). At this time, Nanog expression appears in PGCs only after migration, and down regulated when the PGCs differentiate into adult germ cells (Yamaguchi, Kimura et al. 2005).

According to the evolutionary conservation between homologues in many vertebrates (Jauch, Ng et al. 2008), Nanog in human ES cells has been shown to have the same role in regulation of pluripotency as its mouse counterpart (Hyslop, Stojkovic et al. 2005).

Regulatory sequences in DNA region is a binding site of TFs to become an enhancer or repressor of the genes. In many cell types of vertebrates, TFs orchestrates gene expression by binding in characteristic motifs to procure adequate components of transcriptional machinery.

Nanog in *in vitro* mouse model, epiblast-like cells (EpiLCs) developed from naïve pluripotent embryonic stem (ES) cells cultured in basic fibroblast growth factor (bFGF) and activin A. And also, EpiLCs acquire competence for a PGC-like fate to induce PGC-like cells (PGCLCs) with germline TFs Prdm1, Prdm14 and Tfap2c which are important for PGC

specification(Murakami, Gunesdogan et al. 2016) and bone morphogenetic protein 4 (BMP4) (Chai, Yuen et al. 1999, Hayashi, Ohta et al. 2011). In chicken, *NANOG* is expressed broadly in developmental stage with germ cell restricted expression pattern of protein. Chicken *NANOG* is restrictively expressed in scattered cells over the epiblast only in what seems to have germ cell fate throughout the epiblast at stage HH1 or HH3 (Canon, Herranz et al. 2006).

In result of the studies, *Nanog* is revealed as an important regulator both in pluripotent cells and PGCs. Thus, many studies have been tried to identify the transcriptional regulators to enhance or repress of *Nanog* gene to deeply understand the molecular mechanisms how cell-specific *Nanog* expression is regulated. Thus, in this study, we tried to validate cell specific regions of cells and TFs, and Also, to confirm that epigenetic modification regulates cell specific *Nanog* promoter activities by methylation analysis.

2. Materials and Methods

Experimental Animals and Animal Care

The care and experimental use of chickens were approved by the Institute of Laboratory Animal Resources, Seoul National University (SNU-150827-1). The chickens were maintained according to a standard

management program at the University Animal Farm, Seoul National University, Korea. The procedures for animal management, reproduction, and embryo manipulation adhered to the standard operating protocols of our laboratory.

Collection of chick stage X embryos from eggs

Chick stage X embryos were separated from the egg using sterilized paper (Chapman, Collignon et al. 2001) and the shell membrane and albumen were detached from the yolk. A piece of square-type filter paper (Whatman, Maidstone, Kent, UK) with the hole at the center was placed over the germinal disc. After cutting around the paper containing the stage X embryos, it was gently turned over and transferred to saline buffer to further remove the yolk and the vitelline membrane for embryo collection (Pannett and Compton 1924), and were classified according to the cleavage stages proposed by Eyal-Giladi and Kochav (Eyalgiladi and Kochav 1976). Unfertilized and abnormal embryos were identified by the morphological criteria of blastoderms.

Culture of chicken ES cells

Chicken blastodermal cells from White Leghorn chicken stage X – stage XII blastodermal cells were used (Pain, Clark et al. 1996). Chicken embryonic stem cell (ESC) line was maintained and subcultured with knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS, Hyclone, Utah, USA), 2% (vol/vol) chicken serum (Sigma-Aldrich, St.Louise, MO, USA), 2 mM L-glutamine (Gibco), 1× non-essential amino acids (Gibco), 1× β-mercaptoethanol (Gibco), and 1×Antibiotic-Antimycotic (Gibco, CA, USA). rch LIF (20 ng/mL, Sigma) was used. Cultured cells were serially subcultivated every second or third day to inhibit differentiation by gentle pipetting without any enzyme. The cells were separated gently using the tip of a micropipette and disseminated onto a new plate. Half or all of the incubation medium was replaced daily with fresh medium. Chicken ES cells were cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60-70% relative humidity.

Culture of DF-1 cell line

Chicken DF-1 fibroblast cell line was maintained and subcultured with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) (Hyclone, Utah, USA), and 1X Antibiotic-Antimycotic (Gibco, CA, USA). DF-1 cells were

cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60–70% relative humidity.

Culture of the chicken gonadal PGC line

Primordial germ cells from White Leghorn chicken embryonic gonads at day 6 (stage 28) were maintained and subcultured with knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 20% (vol/vol) Fetal Bovine Serum (FBS, Hyclone, Utah, USA), 2% (vol/vol) chicken serum (Sigma–Aldrich, St.Louise, MO, USA), 1×nucleosides (Millipore, Bedford, MA, USA), 2 mM L–glutamine (Gibco), 1× non–essential amino acids (Gibco), β–mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco), and 1×Antibiotic–Antimycotic (Gibco, CA, USA). Human bFGF (10 ng/mL; Koma Biotech, Seoul, Korea) was used for PGC self–renewal. Chicken PGCs were cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60–70% relative humidity. The cultured PGCs were subcultured onto mitomycin–inactivated MEFs in 5– to 6– day intervals by gentle pipetting without any enzyme treatment (Park and Han 2012).

Construction of the expression vector

For construction of eGFP expression vector, amplified the 5' flanking region of the c*NANOG* gene from genomic DNA of adult chicken were cloned into the pGEM T easy vector (Promega, USA) then, ligated eGFP coding sequence and polyadenylation (Poly-A) tail using restriction enzymes *Spe I* and *Nde I*. As different sizes of the c*NANOG* promoter sequences were cloned, each of PCR products amplified though primer sets (Table 1). The fragment -3,550 bp to +70 bp of chicken *NANOG* gene was cloned from the National Center for Biotechnology Information (NM_001146142.1) for the promoter 5' deletion and fragment assay.

In Vitro Transfection

In vitro transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). For expression analysis of cNANOG, constructed expression vector (5 μg) and 2 ul of Lipofectamine 2000 were separately diluted with 50 ul of Opti-MEM I reduced serum medium (Invitrogen) and incubated at room temperature for 5 min. Liposome-DNA solutions were then mixed and incubated at room temperature for 20 min to form the lipid-DNA complex. Liposome-DNA complex solution was added to 2.5×10^5 cultured PGCs in 500 ul of PGC culture medium. Transfected cells were incubated for 24hr

without feeders. After incubation, cells were analyzed using a fluorescence microscope.

siRNA Transfection in Chicken PGCs

Cells were seeded at a density of 2.5 x 10^5 per well of a 12-well plate in a volume of 1 ml medium. Then, cells were transfected with each siRNAs (50 pmole) with RNAiMAX (Invitrogen, USA). Negative control siRNA that has no complementary sequence in the chicken genome was used as a control. Sequences of each siRNAs are listed in Table 3. After transfection for 48 hr, total RNA was extracted using TRIzol reagent (Invitrogen, USA). The knockdown efficiency of siRNA and their effects on the expression of pluripotency and germ cell-related genes including POUV, NANOG, SOX2, CVH, cDAZL were measured using quantitative RT-PCR.

Quantitative real-time PCR

Total RNA from each samples was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. About 1 μg of total RNA was reverse-transcribed with the Superscript III First-strand Synthesis System (Invitrogen) according to the manufacturer's protocol. The PCR reaction mixture contained 2 μl of PCR buffer, 0.5 μl

of 10 mM dNTP mixture, 10 pmoles each of forward and reverse primers (Table 2 and Table 4), 1 μl of cDNA and 1 U of Taq DNA polymerase in a 20 μl final volume. PCR was performed with initial incubation at 95℃ for 10 min, followed by 40 cycles at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec. PCR was terminated by a a final incubation at the dissociation temperature. The cDNA was used as a template for quantitative real-time PCR which was performed using StepOnePlus real time PCR system (Applied Biosystems, USA) with EvaGreen (Biotium, USA). Each test sample was performed in triplicate. PCR was terminated by a final incubation at the dissociation temperature. Gene expression levels were measured using Quantification of relative gene expression was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = (Ct of the target gene − Ct of GAPDH) stage − (Ct of the target gene − Ct of GAPDH)control.

Statistical Analysis

All data were expressed as mean± S.D. from three independent experiments. One-way ANOVA with Bonferroni compare all pairs of columns was used to calculate the difference between experimental groups. GraphPad Prism v.5 (GraphPad Software, USA) was used to evaluate the data. $P < 0.05$ was considered statistically significant.

Bisulfite Sequencing

Genomic DNA was firstly extracted using Cell lysis solution (CLS)(10% SDS (1%), 1M Tris-HCl pH8.0 (25mM), 500mM EDTA pH8.0 (10mM)) and proteinase K (Quiagen) and Protein precipitation solution (PPS) (7M Ammonium Acetate). and then the DNA was isopropanol-precipitated. DNA was dissolved indifferently in H2O or TE buffer (10 mM Tris-HCl, pH 8.0, and 10 mM EDTA). Bisulfite conversion was performed using the EZ DNA Methylation kit (Zymo Research). Converted DNA was used fresh or stored at -20° C. Converted DNA was amplified by polymerase chain reaction (PCR) by using primers designed with MethPrimer (Table 1). PCR conditions were 95° C for 10 min and 40 cycles of 95° C for 1 min, 49/51/55° C for 1 min (temperature was sample-dependent), and 72° C for 1 min, followed by 10 min at 72° C. PCR products were purified with the Gel and PCR Clean-Up kit (Promega) and then cloned into T vector (Promega) and reverse-sequenced using M13 primers (MWG Biotech, High Point, NC). Methylated sequences were analyze using Bisulfite sequencing DNA Methylation Analysis (BISMA) Tools.

3. Results

From the result of previous study, (Jin 2016) 5' untranslated region of chicken *NANOG* was validated. And also, cNANOG promoter region was studied in two individual regions: distal and proximal regions.

NANOG promoter in chicken has different regulatory mechansisms in cESCs and cPGCs respectively

To determine that cNANOG promoter has cell specific expression in PGCs, we analyzed the expression of cNANOG promoter using cNANOG promoter fragment – GFP expression vector and luciferase assay in both cES cells and cPGCs. Before the analysis of determination, we identified cES cells using alkaline phosphatase (AP), periodic acid Schiff reaction (PAS), SSEA-1. And then, using GFP and luciferase vector, we confirmed that cNANOG promoter has different expression pattern of cNANOG promoter between cell types (Figure 1). This result suggests that cNANOG promoter has different transcription factors and promoter regions depending on the cell types.

The proximal region of the c*NANOG* promoter contains a *cis – regulatory* elementary in the upstream of *core – region*

We predicted common TFs that have binding sites in the 210bp fragment (-130/+70) of the c*NANOG* promoter using genomatix program. Additionally, we attempted to clarify the TFs that were supposed to have a critical role in chicken PGCs than in other cell types, such as Stage X blastodermal cells, gonadal stromal cells (GSCs), and chicken embryonic fibroblasts (CEFs) using previously obtained transcriptome data (Han, Park

et al. 2006, Kim, Park et al. 2007, Lee, Lee et al. 2011). And then, by deletion assay and mutation assay using luciferase assay, we specified the two regions to regulate and initiate the transcription of c*NANOG* promoter. From these analyses, we identified two factors which have putative binding sites in the 12bp fragment (-130/+70 to -108/+70) of the c*NANOG* promoter (Figure 2). To summarize our findings, we marked the consensus sequences and positions of the predicted TFs in sequences of the c*NANOG* promoter including TATA-box sequence.

Transcriptional Factor in Core Proximal Region regulates cNANOG Promoter in chicken PGCs

By using genomatix and transcriptome data, we further sorted transcriptional factors to regulate the transcriptional regulation of NANOG in the 12bp fragment of the cNANOG promoter. As a result, transcriptional binding site of autoimmune regulatory element binding factor (AIRE) and serum response element binding factor (SRF) was contained in this region. Using qRT - PCR, we confirmed the gene expression level of each gene in each cell (Figure 3). The result suggests that SRF might have a role in repression of proximal promoter region in cES and regulates germ cell fate.

The *cis* – *regulatory* elementary of proximal region of the c*NANOG* promoter is hypomethylated in chicken PGCs

To investigate the relationship between gene transcription and methylation in samples, mRNA samples were subjected to RT-PCR. And then, to *Identify the methylated CpG sites in the putative promoters of cNANOG*, we searched for the contiguous sequence of cytosine and guanine in proximal promoter region (Figure). Genomic DNA samples were prepared from Stage X, PGC, and DF1 to analyze their methylation states.

Selection of Transcription factor of Distal region by deletion Assay and Validation by knockdown assay

We also predicted common TFs that have binding sites in the 226bp fragment (-3,154/+70 t0 -2,928/+70) of the c*NANOG* promoter using genomatix program. Additionally, we attempted to clarify the TFs that were supposed to have a critical role in chicken PGCs than in other cell types, such as Stage X blastodermal cells, gonadal stromal cells (GSCs), and chicken embryonic fibroblasts (CEFs) using previously obtained transcriptome data (Han et al., 2006; Kim et al., 2007; Lee et al., 2011).

To determine that POUV in distal region from sorted factors acts as an enhancer, we confirmed the decreased luciferase activity after knockdown of POUV only in promoter contain distal region (Figure 5). This result means

that distal region of the c*NANOG* promoter is regulated by POUV in chicken PGCs

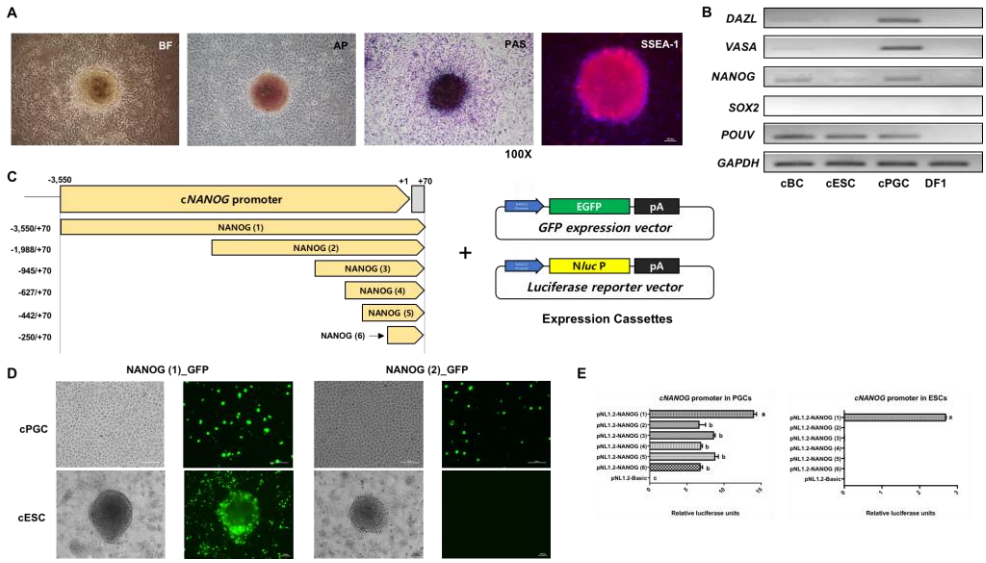


Figure 1. *NANOG* promoter in chicken has different regulatory mechansisms in cESCs and cPGCs respectively (A) Chicken ES cells colonies on a feeder layer of mouse embryonic fibroblasts (×100). (B) RT – PCR pf gene expression of pluripotency and germness related genes in cultured chicken PGCs was monitored by microscopy (C) Overview of the Luciferase and GFP expression vectors that were used. (D) GFP expression of NANOG Promoter_GFP expression vectors in PGCs and ESCs. (D)Expression of NanoLuc luciferase is measured using Nano-Glo-dual luciferase assays in cESCs. Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. Scale bar = 100 μm. Different letters (a-e) indicate significant differences ($P < 0.05$).

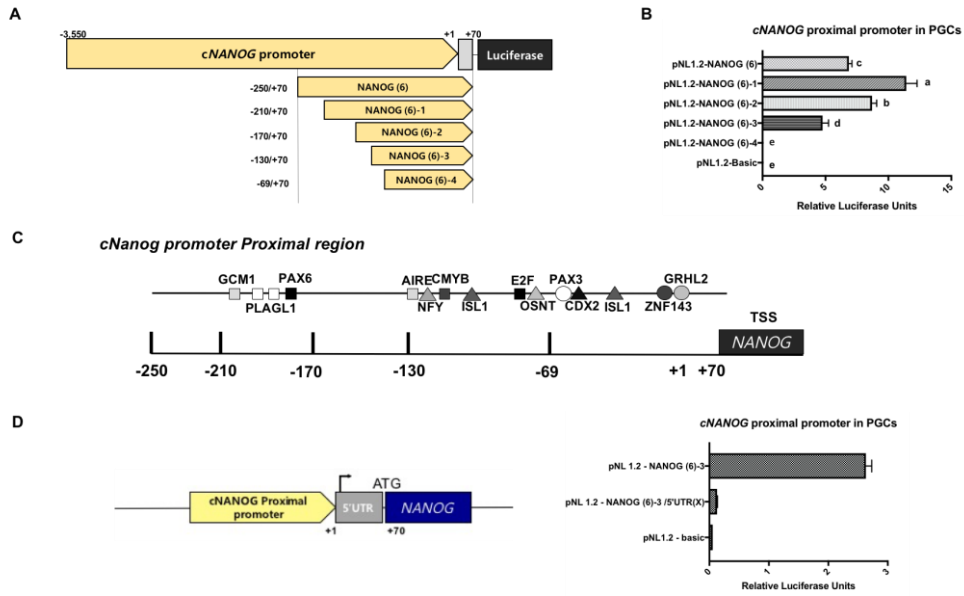


Figure 2. The proximal region of the c*NANOG* promoter contains a *cis – regulatory* elementary in the upstream of *core – region* (A) By 5' deletion assay, four constructs including different lengths of 5' flanking sequences including 5' UTR were analyzed by (B)luciferase assy. (C) Prediction of Transcription factors using Genomatix of Nanog Gene proximal Region (D) Deletion Assay of cNANOG proximal region using Luciferase Assay Deletion assay to validate necessity of 5'UTR cNANOG proximal region.

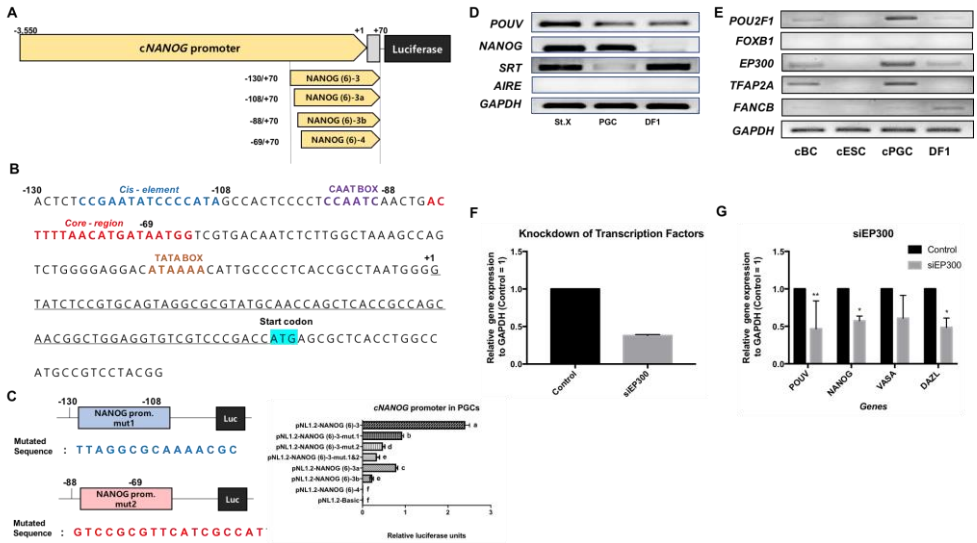


Figure 3. Selection of Transcriptional Factors in Proximal Region regulates core-region and cis-element of cNANOG Promoter in chicken PGCs. (A) Schematic diagram of cis-element and core-region of cNANOG. (B) Nucleic acid sequence of the -130/+70bp chicken NANOG promoter region. (C)Mutation assay of OSNT and AIRE binding site. Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. Scale bar = 100 μm. Different letters (a-e) indicate significant differences ($P < 0.05$). (D) RT - PCR of RNA-seq based selected TFs in each cell type. (E) RT - PCR of TFs predicted in PROMO based on RNA-seq. (F, G) Expression analysis of Knockdown of EP300 by Real-time PCR was conducted in triplicate and normalized to control expression of GAPDH. Significant differences between groups are indicated as ***$P < 0.001$. Error bars indicate the SE of triplicate analyses.

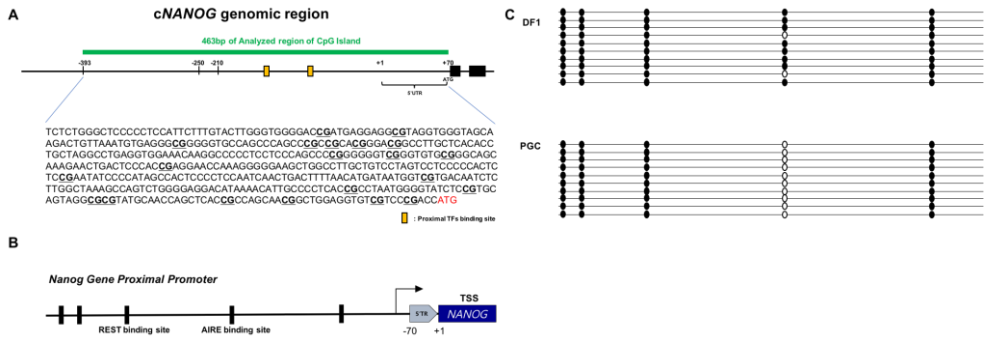


Figure 4. Methylation Analysis of proximal region shows that c*NANOG* promoter is hypomethylated in chicken PGCs (A) Schematic illustration of the methylation analysis of the proximal region of *Nanog*. Green shaded indicates the region used for bisulphite genomic sequencing analysis. CpG islands indicates by emphasis and underscore. (B)CpG islands on −130/+70 region of cNANOG promoter. (C) Methylation patterns of stage X, PGC, and DF1.

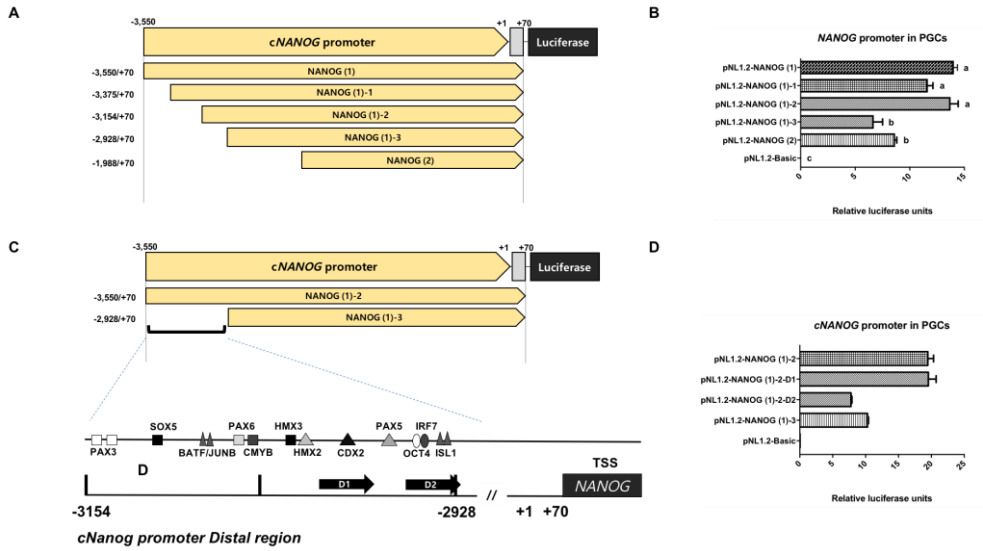


Figure 5. Selection of Transcription factor of Distal region by deletion Assay and Validation by knockdown assay (A) Deletion Assay of six fragments of Distal region were analyzed by (B)luciferase assy. (C) Schematic diagram of prediction of transcription factors was performed using MatInspector software in distal region of NANOG promoter (D)Validation of deletion assay of distal regions of PGCs. Promoter activity was measured as the ratio of NanoLuc to firefly and normalized for transfection efficiency by using control firefly luciferase expression. Scale bar = 100 μm. Different letters (a–c) indicate significant differences ($P < 0.05$).

Table 1. List of primer sequences for cloning of the *cNANOG* promoter using genomic PCR

Primer set	Primer sequence (5' → 3')
5'tr deletion forward - F	CCCATTAGGCGGTGAGGGGCAATGTTTTATGTCCTCCCCAGA
pNL1.2 vector R	CTCGAGGATATCAAGATCTGGCCTCGGCGGCCAAGCT T
PGC Distal (1) - F	CACAGATGAGTCACGTTTGAGTCAGTTGCAGAAGAACACTTAATCTAC
PGC Distal (2) - F	AATCTACATAAAATTTGAGACACCTCTACCAGCGTAACAGTGACCCACTG
PGC Distal (3) - F	ACCCACTGATGAAAATGAACTTAGCAGTGCTACGAGGTAGGAACCC
pNL1.2(KpnI) - R	GGTACCGGCCAGTTAGGCCAGAGAAATGTTC
ESC Distal (1) - F	GTTCCCCTAAAAAGTCAAGAG
ESC Distal (2) - F	TTCTTTGTTGGCTGCCTTCCTTCCA
ESC Distal (3) - F	TTGATCCAGACTGCTAGCTGTGAC
Bisulfite - F	TTGTATTTGGGTGGGGATCGATGAGGAGGCGTAG
Bisulfite - R	CTTCCGCGACAAACGCGCTATCCATACTAC

Table 2. List of primers used for quantitative real-time PCR

No.	Gene Symbol	Description	Accession No.	Primer sequence (5' → 3')
1	SRF	serum response factor	NM_001252141.1	F: GAGCACATCTTCAAGGAGAC
				R: CTTTTCTCTTGCCTTGGGTT
2	AIRE	autoimmune regulator-like	EU030007.1	F: GTTCCCCATCACGAATTACC
				R: CTGGGAGAGGTCTGAGTAAG
3	FOXB1	forkhead box B1	XM_004943754.2	F: ATC ATG GAC CGT TTC CCC TA
				R: TTG TAG GTG GAC ATC TGG GG
4	POU2F1	POU class 2 homeobox 1	ENSGALG00000015446	F: GCT TGA TGG CAT CCT CAC AG
				R: AGG AAC AGA GGG GCA GTT AC
5	FANCB	Fanconi anemia complementation group B	ENSGALG00000016569	F: ACG TCT TGG CCT ACA ATG GA
				R: CGA CGC CAT TGT GCT TTT TC
6	TFAP2A	transcription factor AP-2 alpha	ENSGALG00000012775	F: CTT CAA CCT CAT CTC GCA CG
				R: CTC ATT CTG TTG CCG TAG CC

4. Discussion

Transcriptional regulation using TFs on the promoter is the issue among the early embryo and germ cell developmental studies. Tissue-specific gene expression is orchestrated by interactions between TFs and *cis*-regulatory elements (Spitz and Furlong 2012).

Nanog, the homeodomain transcription factor, which has crucial role in both pluripotent stem cells and germ cells. Also, Nanog in chicken was identified to express restricted expression in PGCs after HH3 (Canon, Herranz et al. 2006).

In the present study, we identified the different expression patterns of distal and proximal regions, respectively in cultured chicken ESCs and PGCs. By the results of dual luciferase reporter assay, chicken *NANOG* gene is express in both cells in promoter contains distal region, but proximal promoter region. To determine the transcriptional regulators of NANOG gene, many studies to figure out TFs have been proceeded in many species, such as Oct4, Sox2.

In mouse *Oct4* gene (Yeom, Fuhrmann et al. 1996), two separate regulatory elements regulate gene expression depend on the temporal and spatial during embryonic development. The distal element is specifically active in embryonic stem and embryonic germ cells. In similar to chicken *NANOG* gene, the proximal enhancer is only active in the epiblast of mouse embryos with stage-specific manner.

In our study, NANOG expression patterns of PGCs are different with cESCs. We analyzed the transcription factors and methylation patterns, in the views of transcriptional and epigenetic, respectively. To confirm the factors, knockdown of each factor was implemented.

Many factors predicted from our studies are related to TGF-β signaling pathways. Thus, this data suggested that germ cell characteristics are the result of TGF-β signaling pathways (Whyte, Glover et al. 2015), similar to bFGF signaling pathway (Choi, Kim et al. 2010).

CHAPTER 4.
Epigenetically regulated moderate level of NANOG maintains PGC integrity in chicken PGCs by CoREST/HDAC Corepressor complex

1. Introduction

Primordial germ cells (PGCs), the precursors of sperm and oocytes which contains genetic information of organisms, are regulated by coordinated actions such as Transcription factors (TFs), RNA binding proteins (RBPs), and Epigenetic modifications to acquire germ cell specification, migration and the ability to differentiate into mature germ cells. Chromatin epigenetic modifications, such as DNA methylation and histone post-translational modifications (PTMs) have a role in regulation of cell characteristic such as pluripotency and germness in PGCs. During early development in many vertebrates, the patterns of DNA methylation and the associated chromatin remodeling are the crucial steps to undergo developmental steps of PGCs (Arand, Wossidlo et al. 2015, Kurimoto, Yabuta et al. 2015, Walter, Teissandier et al. 2016).

Recent studies find that epigenetic regulations like loss of 5mC in whole genome, DNA methylation, histone and chromatin modifications have a crucial role in PGCs to eliminate paternal imprinting in mammals (Kawasaki, Lee et al. 2014). However, studies of histone and chromatin modifications and also the control of epigenetic patterns in chicken are highly limited. In the previous studies, methylation and acetylation during germ cell specification to differentiation activate germ cell specific genes and repress somatic cell genes in mouse. And also, in chicken, the regulation is regulated epigenetically. In contrast to mammals, the H3K27me3 global level

is reduced, whereas the H3K9me3 level is increased in chicken. However, other epigenetic modifications such as the level of acetylation/deacetylation level in PGCs still yet unknown in vertebrates.

Acetylation/deacetylation regulates gene expressions by relaxation or condensation of chromatin structure by Histone acetyltransferase (HAT) and Histone deacetylase (HDAC), respectively (Grozinger and Schreiber 2002). CoREST, NURD, and SIN3A complexes, the three major Class I HDAC complexes are abundantly existed in cells which have pluripotency. The CoREST complex in both mouse and human has been shown to establish and maintain pluripotency in the formation of iPS cells (Yang, Wang et al. 2011), and the SIN3A/HDAC complex in mouse ESCs increase transcriptional functions with NANOG and also reprogramming efficiency. This means that HDAC can co-localize and co-activate with NANOG to efficiently regulated transcriptional programs at the chromatin level (Saunders, Huang et al. 2017). The CoREST ([co]repressor for element-1 silencing transcription factor) complex was firstly identified in mammals to be associated with the repressor for element-1 silencing transcription factor (REST)/neuronal restrictive silencing factor to have a crucial role in regulating neuronal gene expression and neuronal stem cell fate (Chong, Tapia-Ramirez et al. 1995). CoREST proteins are conserved chromatin modifying complexes composed of integral subunits. REST is broadly expressed in mouse early development

(Chen, Paquette et al. 1998, Grimes, Nielsen et al. 2000). Mouse CoREST mRNA at E8.5 strongly expressed in head mesenchyme and becomes to have an omnipresent expression by E11.5 (Grimes, Nielsen et al. 2000). Using Knockout model of REST in mouse, REST was validated to regulate retarded cell growth and also the death of widespread apoptotic cells in E9.5 to E11.5 (Chen, Paquette et al. 1998) These complexes combine *histone demethylase and deacetylase and have been identified in* many species, such as *Drosophila* (Dallman, Allopenna et al. 2004), *C. elegans* (Smialowska and Baumeister 2006) and mammals (Cowger, Zhao et al. 2007). CoREST complex is also associated with lincRNA HOTAIR in breast cancer (Gupta, Shah et al. 2010, Tsai, Manor et al. 2010). These results suggest that CoREST might have a role in germ cells. NANOG also has a crucial role in embryonic and germline development, and also establish naïve pluripotency in the final reprogramming stage (Saunders, Faiola et al. 2013).

In this study, we demonstrate that CoREST /HDAC complex regulates *NANOG* in chicken PGCs with deacetylation of H3K9Ac. We further confirm that maintenance of moderate NANOG level regulates PGC integrity to differentiate into functional gametes. The result of this study demonstrates that chicken has specific epigenetic regulation during PGC development.

2. Materials and methods

Experimental animals

White Leghorn (WL) hens (54-56 weeks old) were used for the collection of PGCs. We managed chickens according to our standard operation protocol. Relevant experimental procedures for the study were approved by the Institutional Animal Care and Use Committee, Seoul National University before undertaking experiments (SNU-070823-5).

Culture of DF-1 cell line

Chicken DF-1 fibroblast cell line was maintained and subcultured with Dulbecco's modified Eagle's medium (DMEM) (Hyclone, Utah, USA) supplemented with 10% (vol/vol) Fetal Bovine Serum (FBS) (Hyclone, Utah, USA), and 1X Antibiotic-Antimycotic (Gibco, CA, USA). DF-1 cells were cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60-70% relative humidity.

Culture of the chicken gonadal PGC line

Primordial germ cells from White Leghorn chicken embryonic gonads at day 6 (stage 28) were maintained and subcultured with knockout Dulbecco's Modified Eagle's Medium (DMEM, Gibco, CA, USA) supplemented with 20% (vol/vol) Fetal Bovine Serum (FBS, Hyclone, Utah, USA), 2% (vol/vol) chicken serum (Sigma-Aldrich, St.Louise, MO, USA),

1×nucleosides (Millipore, Bedford, MA, USA), 2 mM L-glutamine (Gibco), 1× non-essential amino acids (Gibco), β-mercaptoethanol (Gibco), 1 mM sodium pyruvate (Gibco), and 1×Antibiotic-Antimycotic (Gibco, CA, USA). Human bFGF (10 ng/mL; Koma Biotech, Seoul, Korea) was used for PGC self-renewal. Chicken PGCs were cultured in an incubator at 37℃ with an atmosphere of 5% CO_2 and 60-70% relative humidity. The cultured PGCs were subcultured onto mitomycin-inactivated MEFs in 5- to 6- day intervals by gentle pipetting without any enzyme treatment (Park and Han 2012).

Construction of the expression vector

For construction of eGFP and cNANOG expression vector, amplified the CDS region of genes from cDNA of chicken PGCs and previously constructed vector were cloned into the pGEM T easy vector (Promega, USA) then, ligated coding sequence and polyadenylation (Poly-A) tail using restriction enzymes ECoRI. Each CDS sequences are ligated into pCE - hSK (commercially availavable on Addgene).

In Vitro Transfection

In vitro transfection was performed using Lipofectamine 2000 according to the manufacturer's instructions (Invitrogen, USA). For expression analysis of eGFP and cNANOG, constructed expression vector (5 μg) and 2 ul of Lipofectamine 2000 were separately diluted with 50 ul of

Opti-MEM I reduced serum medium (Invitrogen) and incubated at room temperature for 5 min. Liposome-DNA solutions were then mixed and incubated at room temperature for 20 min to form the lipid-DNA complex. Liposome-DNA complex solution was added to 2.0 x 10^5 cultured PGCs in 500 ul of PGC culture medium. Transfected cells were incubated for 24hr without feeders. After incubation, cells were analyzed using a fluorescence microscope.

siRNA Transfection in Chicken PGCs

Cells were seeded at a density of 2.0 x 10^5 per well of a 12-well plate in a volume of 1 ml medium. Then, cells were transfected with each siRNAs (100 pmole) with RNAiMAX (Invitrogen, USA). Negative control siRNA that has no complementary sequence in the chicken genome was used as a control. Sequences of each siRNAs are listed in Table 3. After transfection for 48 hr, total RNA was extracted using TRIzol reagent (Invitrogen, USA). The knockdown efficiency of siRNA and their effects on the expression of pluripotency and germ cell-related genes including POUV, NANOG, SOX2, CVH, cDAZL were measured using quantitative RT-PCR.

Quantitative Real Time-PCR

Total RNA from each sample was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. About 1 μg of total RNA was reverse-transcribed with the Superscript III

First-strand Synthesis System (Invitrogen) according to the manufacturer's protocol. The PCR reaction mixture contained 2 μl of PCR buffer, 0.5 μl of 10 mM dNTP mixture, 10 pmoles each of forward and reverse primers (Table 2 and Table 4), 1 μl of cDNA and 1 U of Taq DNA polymerase in a 20 μl final volume. PCR was performed with initial incubation at 95℃ for 10 min, followed by 40 cycles at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec. PCR was terminated by a final incubation at 72℃ for 5 min. The cDNA was used as a template for quantitative real-time PCR which was performed using StepOnePlus real time PCR system (Applied Biosystems, USA) with EvaGreen (Biotium, USA). Each test sample was performed in triplicate. PCR was terminated by a final incubation at the dissociation temperature. Gene expression levels were measured using Quantification of relative gene expression was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct$ = (Ct of the target gene - Ct of GAPDH) stage -(Ct of the target gene - Ct of GAPDH)control.

Statistical Analysis

All data were expressed as mean± S.D. from three independent experiments. One-way ANOVA with Bonferroni compare all pairs of columns was used to calculate the difference between experimental groups. GraphPad Prism v.5 (GraphPad Software, USA) was used to evaluate the data. $P < 0.05$ was considered statistically significant.

Immunocytochemistry

Cells were washed twice in PBS and fixed with 4% paraformaldehyde for 20 min. After permeabilization with 0.1% Tween-20 and 1% Triton-X, non-specific binding was blocked with 1% normal goat serum. Embryos were then incubated with a rabbit anti-acetyl-histone H3K9 antibody (Abcam, ab61231) at 4 ° C overnight. Cells incubated in the absence of primary antibody were used as a negative control. After extensive washing, embryos were incubated with secondary antibodies Goat Anti-Rabbit IgG H&L (Alexa Fluor® 594) (Abcam, ab150080), for 1 h. After washing, cells were mounted in a drop of DAPI and examined using a confocal microscope (Nikon Co. Kanagawa, Japan). The evaluation of fluorescence intensity in relation to nuclear size was performed with the EZ-C1 FreeViewer software (Nikon) using arbitrary fluorescence unit. All images were background corrected in comparison with the background of corresponding negative controls.

Chromatin Immunoprecipitation

All cell types were fixed in 1% methanol-free formaldehyde (Thermo Scientific, 28906) in D-MEM for 5 min at room temperature, followed by 10 min blocking in 125 mM glycine. Cells were rinsed two times in ice-cold PBS and pelleted (1070rpm, 4 min, 4° C). Suspension cultured cells were fixed in the plate and harvested by pippetting after PBS

washes. All fixed cell pellets were dissociated in RIPA lysis buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 0.5 M EGTA, 1% Triton X-100, 0.1% SDS, 0.1% Na-deoxycholate, and 140 mM NaCl; Thermo, Waltham, MA, USA) with protease inhibitor (Sigma, St. Louis, MO, USA) and phosphatase inhibitor (Sigma, St. Louis, MO, USA). ChIP kits (ab117138) were purchased from Abcam. ChIP was conducted following the instructions of vendor. Primers used for CHIP -qPCR and PCR (Table)

CHIP - qPCR and RT PCR

The PCR reaction mixture contained 2 μl of PCR buffer, 0.5 μl of 10 mM dNTP mixture, 10 pmoles each of forward and reverse primers (Table 2 and Table 4), 1 μl of cDNA and 1 U of Taq DNA polymerase in a 20 μl final volume. For Quantitative Real Time PCR was performed with initial incubation at 95℃ for 10 min, followed by 40 cycles at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec. PCR was terminated by a final incubation at 72℃ for 5 min. The cDNA was used as a template for quantitative real-time PCR which was performed using StepOnePlus real time PCR system (Applied Biosystems, USA) with EvaGreen (Biotium, USA). RT - PCR was performed with initial incubation at 95℃ for 10 min, followed by 30 cycles at 95℃ for 30 sec, 60℃ for 30 sec and 72℃ for 30 sec. PCR was terminated by a final incubation at 72℃ for 5 min. The default Input fraction is 1% which is a dilution factor (DF) of 6.644 cycles (i.e. $\log_2$ of 100), NS=Non-Specific Ab (Mu/Rb IgG).

Gene expression levels were measured using Quantification of relative gene expression was calculated using the following formula: $2^{-\Delta\Delta Ct}$, where $\Delta\Delta Ct = (\triangle Ct[IP] - \triangle Ct[NS])$.

Western blot

Crude protein was isolated from 2 x 10^6 cells of samples by dissociation in RIPA lysis buffer (Thermo, Waltham, MA, USA) with protease inhibitor (Sigma, St. Louis, MO, USA) and phosphatase inhibitor (Sigma, St. Louis, MO, USA). Approximately 5ug of protein from total cell lysate was used in each lane for separation in a 10% SDS-PAGE gel. The protein was transferred onto a Hybond 0.45 PVDF membrane (GE Healthcare Bio-sciences, Little Chalfont, UK), and blocked with 3% skim milk for one hour at room temperature (Sigma, St. Louis, MO, USA). Subsequently, the blocked membrane was incubated overnight at 4° C for primary antibody attachment with 1:1,000 dilution. Horseradish peroxidase conjugated secondary antibody (Thermo, Waltham, MA, USA) was attached with 1:4,000 dilutions at room temperature for one hour. The primary antibodies used were as the following: anti-VASA and anti- α - globulin (Santa Cruz, Santa Cruz, CA, USA). The expression was visualized using ECL western blotting detection system (GE Healthcare Bio-sciences, Little Chalfont, UK).

3. Results

Nanog regulates cell characteristics in chicken PGCs

The gene expression of pluripotency and germ cell related genes of PGCs was measured by qRT－PCR. By using qRT-PCR analysis after overexpression and knockdown of NANOG, the results indicated that NANOG regulates genes expression of pluripotency and germness to have a role in regulation of proliferation related to pluripotency and germness (Figure 1).

Validation of epigenetic modification markers in chicken PGCs

To study epigenetic regulation of chicken PGCs, we confirmed in vitro PGC cell line with germ cell markers VASA with co-staining of SSEA-1, and also preliminarily validated methylation and acetylation marker. Staining of Acetylation Marker H3K9ac and H3K27ac and methylation marker H3K9me3 and H3K27me3 with co-staining of SSEA-1 (Figure 2).

Knockdown of REST Results in an Increase of the gene level of NANOG

Transcription factor REST was predicted to have a binding site on *NANOG* promoter upstream region predicted by PromoterInspector

(Genomatix). The gene expression of pluripotency and germ cell related genes of PGCs was measured by qRT-PCR. By using qRT-PCR analysis after overexpression and knockdown of NANOG, the results indicated that NANOG regulates genes expression of pluripotency and germness to have a role in regulation of proliferation related to pluripotency and germness (Figure 3E).

To analyze whether *REST* regulates the level of H3K9Ac to control transcriptional level of *NANOG*, the formaldehyde-cross-linked chromatin fragments from PGCs were chromatin-immuno-precipitated with the anti-H3K9Ac antibody. To validate chromatin enrichment of the region, ChIP-PCR assay was performed using primers located in the promoters of *REST* binding sites on *NANOG* promoter predicted by PromoterInspector (Genomatix) (Fig. 3A). Primers located upon *NANOG* promoter was used as a non-target site for *REST* binding. The promoter region of *REST* binding site was amplified from H3K9Ac immuno-precipitated DNA, confirming chromatin enrichment by ChIP, whereas non-target site used for negative control weakly amplified. In addition, ChIP-PCR assay using Target site primer of knockdown of *REST* sample compared to control shows higher amplification. Thus, based on the result of ChIP PCR, *REST*, a well-known co-repressor of *CoREST* complex, changes the level of PGC specific H3K9Ac (Fig. 3F,G).

RCOR3, the CoREST complex member, orchestrates germ cell specific epigenetic remodeling to repress NANOG gene expression in PGCs

To elucidate further which HDAC complex is related on the change of acetylation in PGCs associated with *NANOG* gene upregulation by co-regulation with *REST*, we first determined the member of each HDAC complexes in chicken blastoderm cell(BC)s, chicken PGCs, and DF1 using RT-PCR of predicted HDAC complex based on RNA-seq data of chicken (Fig. 4A). In the result of RT-PCR based on RNA-seq data, HDAC complexes except SIN3A in PGCs were all expressed. Thus, we validated that RCOR3, the member of CoREST complex (Barrios, Gomez et al. 2014), regulates pluripotency-related gene, *NANOG,* in PGCs by knockdown of the members of each HDAC complex (Fig. 4B, C). Taken together, our findings suggested that *NANOG,* ESC-core transcription factor and germ cell inducing factor in mice (Tang, Kobayashi et al. 2016) is orchestrated by CoREST-HDAC complex.

HDAC inhibitor VPA and Romidepsin treatment of chicken PGCs

Chemical optimization was previously proceeded (Figure 5). To verify that REST regulates *NANOG* as HDAC 1/2 complexes, we analyzed the gene expression by qRT-PCR after treatment of global hdac inhibitor valproic acid (VPA) and HDAC 1 and 2 inhibitor by concentration

difference of it, romidepsin (Figure 5). Both optimized VPA and Romidepsin shows the change of gene expression. Especially, only NANOG and VASA expression is rapidly upregulated after romidepsin. The results indicated that REST-HDAC1/2 compelexes especially regulates NANOG and VASA.

Epigenetically regulated NANOG controls VASA in chicken PGCs

NANOG binding sites were observed in the chicken vasa homologous (*CVH*) promoter. Thus, we analyzed the binding site of NANOG with fragmentation assay of CVH after overexpression and knockdown of NANOG. The results indicated that NANOG binding on -756bp of CVH promoter regulates VASA. In addition to the analyze of binding site, the translation of VASA protein was measured by western blot after knockdown of NANOG and treatment of HDAC inhibitor (Figure 6).

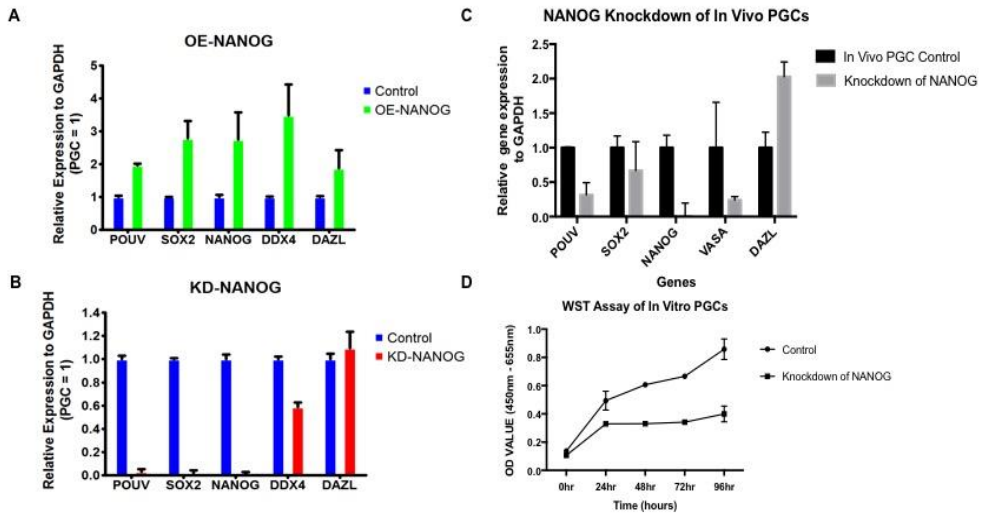


Figure 1. Relative gene expression analysis after overexpression and knockdown of predicted transcription factors in vivo and in vitro cultured PGCs. Chicken primordial germ cells were cultured or immediately MACS sorted of taken after 55hr incubation at 37° C. The relative expression analysis of pluripotency and germ cell-related genes in vivo and cultured PGCs after treatment of overexpression vector and siRNA. GAPDH was used as a control for silencing specificity of the knockdown probes. qPCR was conducted in triplicated, normalizing data to control expression of GAPDH. For statistical analysis, one-way ANOVA test was used and values were compared to control. ***p value < 0.001, **p value < 0.01, *p value < 0.05

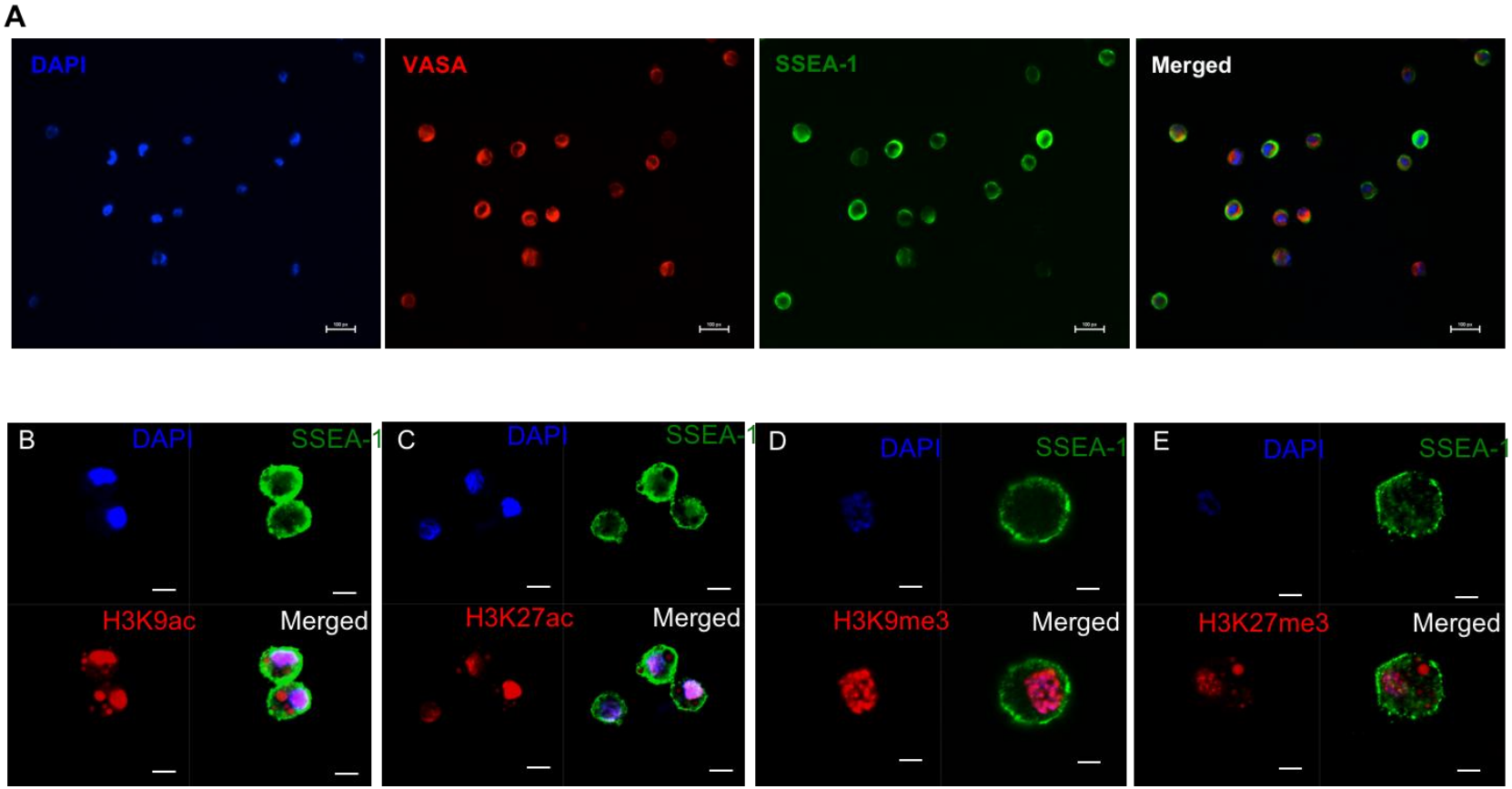

A
DAPI
VASA
SSEA-1
Merged
B
DAPI
SSEA-1
H3K9ac
Merged
C
DAPI
SSEA-1
H3K27ac
Merged
D
DAPI
SSEA-1
H3K9me3
Merged
E
DAPI
SSEA-1
H3K27me3
Merged

Figure 2 . Epigenetic regulation in chicken primordial germ cells. (A) Immunocytochemistry to validate epigenetic marker of In Vitro Primordial germ cells. Staining of Acetylation Marker (B) H3K9ac and (C) H3K27ac and methylation marker (D) H3K9me3 (E) H3K27me3. Scale bar is 100 μm.

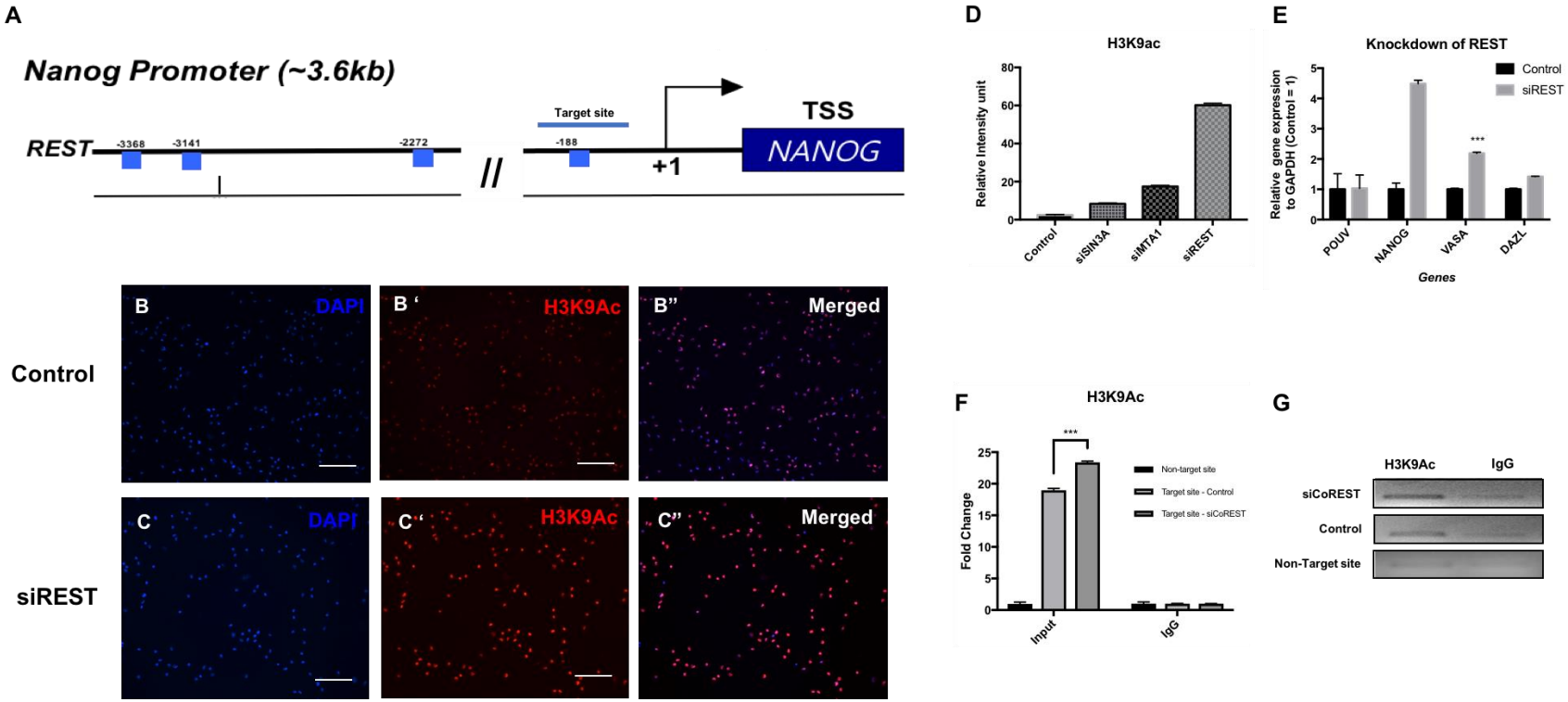

A
Nanog Promoter (~3.6kb)
REST
-3368
-3141
-2272
Target site
-188
+1
TSS
NANOG
Control
siREST
B
DAPI
B '
H3K9Ac
B"
Merged
C
DAPI
C'
H3K9Ac
C"
Merged
D
H3K9ac
Relative Intensity unit
Control
siSIN3A
siMTA1
siREST
E
Knockdown of REST
Relative gene expression to GAPDH (Control = 1)
Control
siREST
POUV
NANOG
VASA
DAZL
Genes
F
H3K9Ac
Fold Change
Non-target site
Target site - Control
Target site - siCoREST
Input
IgG
G
H3K9Ac
IgG
siCoREST
Control
Non-Target site

Figure 3. REST regulates H3K9Ac to upregulate NANOG in chicken PGCs. (A)Target sites of CHIP-qPCR of NANOG Promoter. (B-C) Immunocytochemistry performed on chicken PGCs after siRNA treatment of REST. Scale bars are 50 μm. (D) Relative Intensity unit analysis of samples using EZ-C1 FreeViewer software (Nikon) (E) Expression analysis of Knockdown of HDAC Complexes by Real-time PCR was conducted in triplicate and normalized to control expression of GAPDH. Significant differences between groups are indicated as ***P < 0.001. Error bars indicate the SE of triplicate analyses. (F-G) Chromatin enrichment was confirmed by PCR amplification of the promoters of c*NANOG* and the band was validated by gel electrophoresis. NANOG Promoter upstream region which is not expected to bear REST-binding sites, was used as negative control. Inp, 0.2% input DNA.

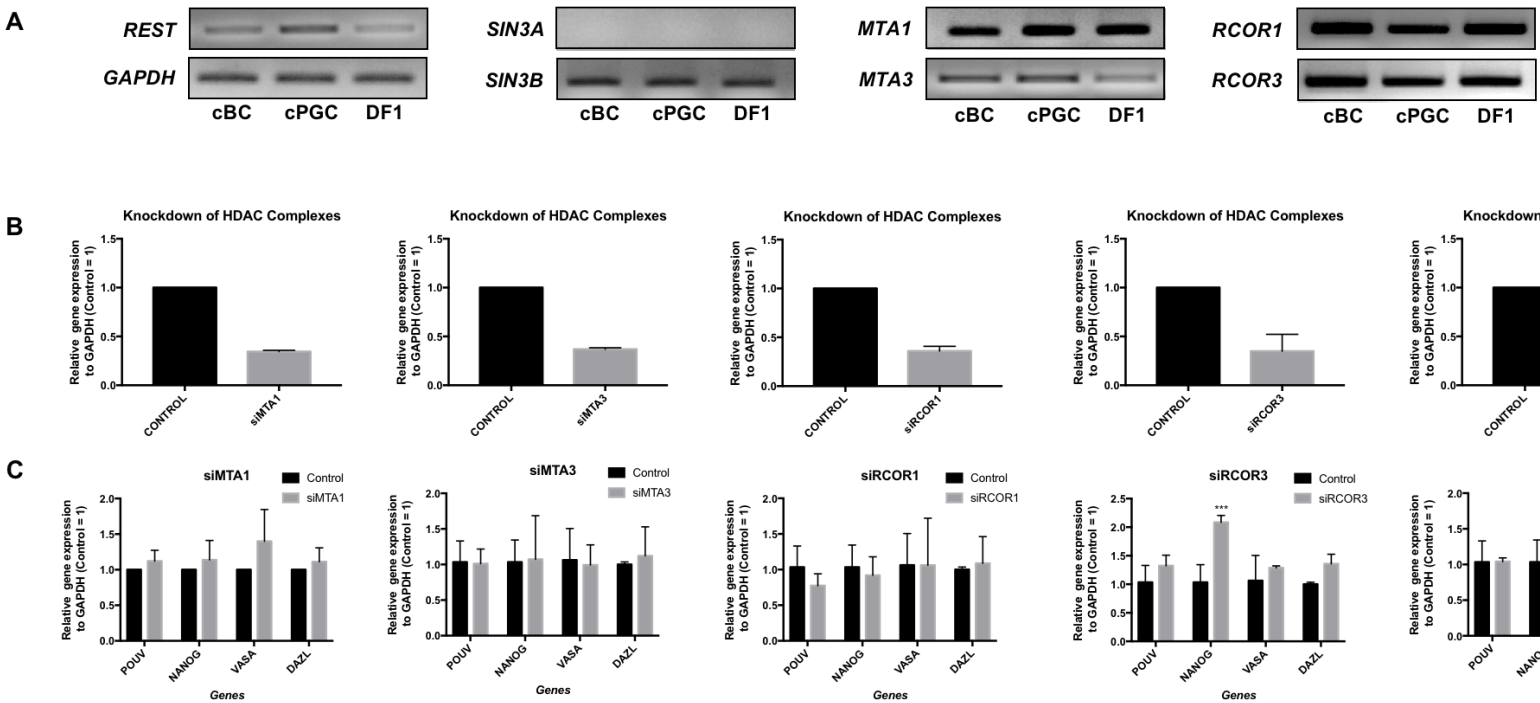
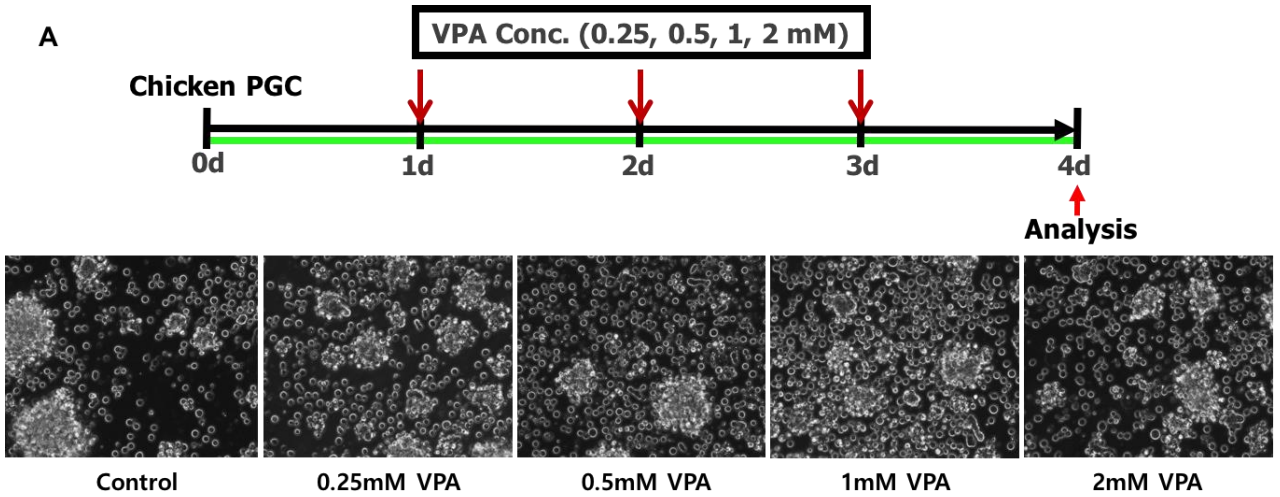
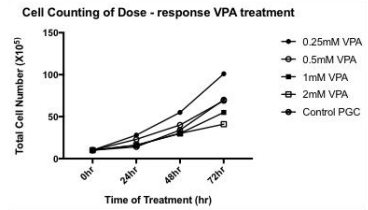
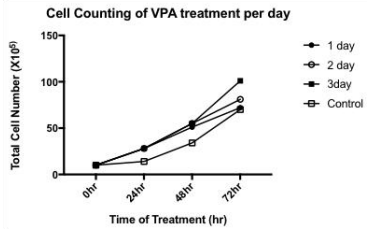
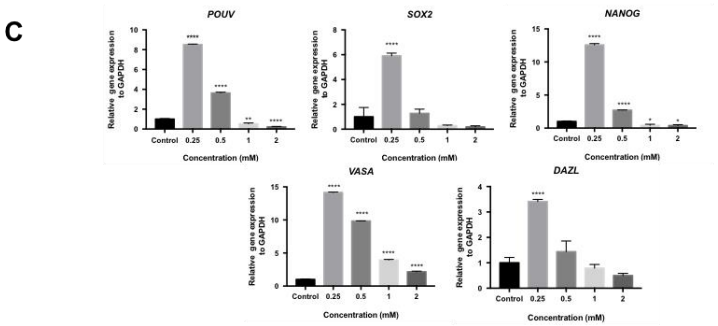

A
REST
GAPDH
cBC cPGC DF1
SIN3A
SIN3B
cBC cPGC DF1
MTA1
MTA3
cBC cPGC DF1
RCOR1
RCOR3
cBC cPGC DF1
B
Knockdown of HDAC Complexes
Relative gene expression to GAPDH (Control = 1)
CONTROL
siMTA1
Knockdown of HDAC Complexes
CONTROL
siMTA3
Knockdown of HDAC Complexes
CONTROL
siRCOR1
Knockdown of HDAC Complexes
CONTROL
siRCOR3
Knockdown of HDAC Complexes
CONTROL
siSIN3B
C
siMTA1
Control
siMTA1
siMTA3
Control
siMTA3
siRCOR1
Control
siRCOR1
siRCOR3
Control
siRCOR3
***
siSIN3B
Control
siSIN3B
Relative gene expression to GAPDH (Control = 1)
POUV
NANOG
VASA
DAZL
Genes

Figure 4. ***RCOR3, the CoREST complex member, orchestrates germ cell specific epigenetic remodeling to repress NANOG gene expression in PGCs.*** Expression analysis of HDAC complexes in chicken primordial germ cells (PGCs) by RT PCR(A). (D) Expression analysis of Knockdown of HDAC Complexes by Real-time PCR was conducted in triplicate and normalized to control expression of GAPDH. Significant differences between groups are indicated as ***P < 0.001. Error bars indicate the SE of triplicate analyses.


A
VPA Conc. (0.25, 0.5, 1, 2 mM)
Chicken PGC
0d
1d
2d
3d
4d
Analysis
Control
0.25mM VPA
0.5mM VPA
1mM VPA
2mM VPA


B
Cell Counting of Dose - response VPA treatment
Total Cell Number (X10^5)
Time of Treatment (hr)
0hr
24hr
48hr
72hr
0.25mM VPA
0.5mM VPA
1mM VPA
2mM VPA
Control PGC


Cell Counting of VPA treatment per day
Total Cell Number (X10^5)
Time of Treatment (hr)
0hr
24hr
48hr
72hr
1 day
2 day
3day
Control


C
POUV
SOX2
NANOG
VASA
DAZL
Relative gene expression to GAPDH
Concentration (mM)
Control
0.25
0.5
1
2

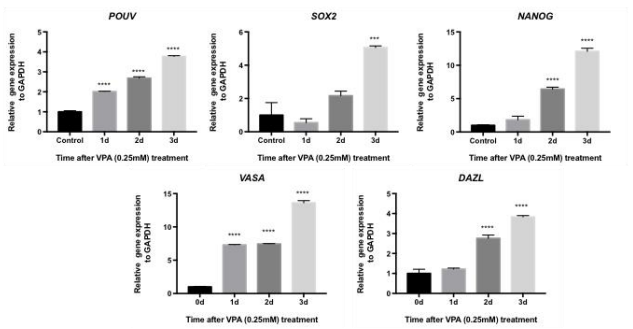
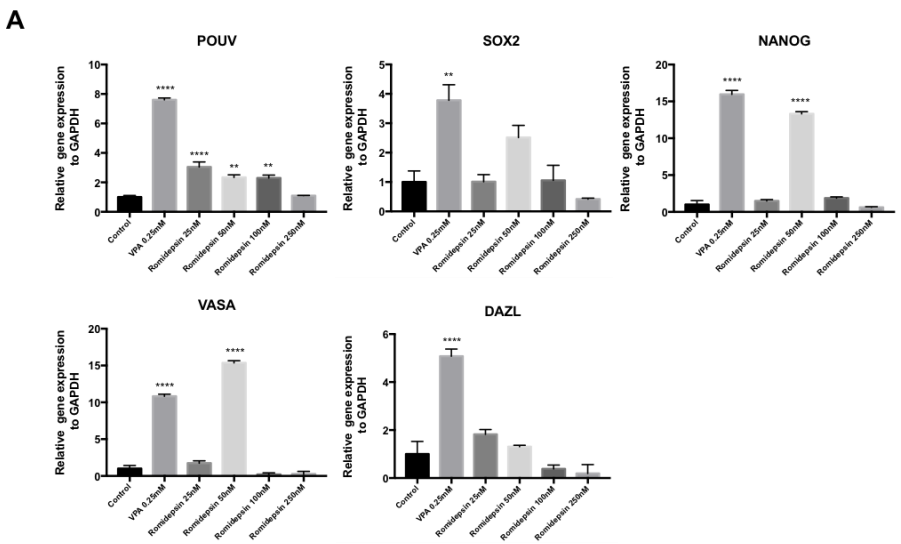
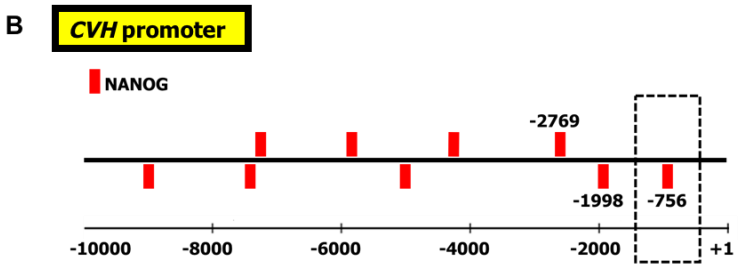
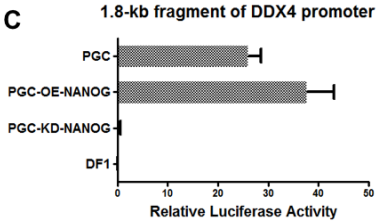

D
POUV
SOX2
NANOG
VASA
DAZL
Relative gene expression to GAPDH
Time after VPA (0.25mM) treatment
Control
1d
2d
3d

Figure 5. Optimization of HDAC inhibitor treatment in vitro chicken PGC (A) Scheme of Dose- and Time -response curve of VPA treatment in chicken PGCs and the Morphology of Control and VPA treated PGCs (B) Optimal Concentration for treatment of Valproic acid is 0.25mM and Cell counting for whole day of passage shows the highest Total Cell Number (C-D) Dose - response of Chemical Treatment. qRT PCR analysis of pluripotency and germness-related genes expression of Dose- and Time-response treatment of VPA to Primordial Germ Cells. For statistical analysis, the one-way ANOVA test was used and values were compared to Control PGC. ****p value $\leq$ 0.0001, ***p value $\leq$ 0.001, **p value $\leq$ 0.01, *p value $\leq$ 0.05.


A
POUV
SOX2
NANOG
VASA
DAZL
Relative gene expression to GAPDH
Control
VPA 0.25mM
Romidepsin 25nM
Romidepsin 50nM
Romidepsin 100nM
Romidepsin 250nM


B
CVH promoter
NANOG
-2769
-1998
-756
-10000
-8000
-6000
-4000
-2000
+1


C
1.8-kb fragment of DDX4 promoter
PGC
PGC-OE-NANOG
PGC-KD-NANOG
DF1
Relative Luciferase Activity

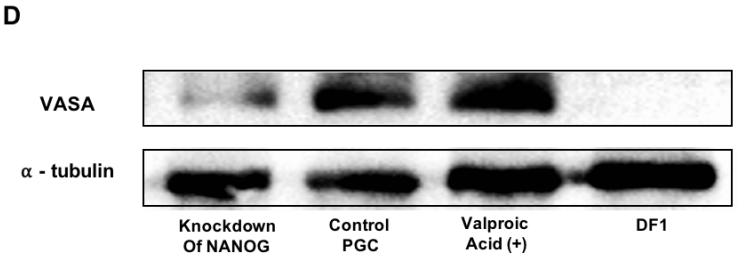

D
VASA
α - tubulin
Knockdown Of NANOG
Control PGC
Valproic Acid (+)
DF1

Figure 6. Gene expressions of NANOG and VASA are upregulated by inhibition of Histone deacetylase by VPA and romidepsin treatment. (A) Scheme of Dose- and Time -response curve of VPA treatment in chicken PGCs and the Morphology of Control and VPA treated PGCs. For statistical analysis, the one-way ANOVA test was used and values were compared to Control PGC. ****p value $\leq$ 0.0001, ***p value $\leq$ 0.001, **p value $\leq$ 0.01, *p value $\leq$ 0.05. (B) NANOG expression pattern analysis by RNA-seq and VASA promoter prediction (~10kb) of the upstream region of PGC specific VASA promoter contains NANOG binding site. Prediction was proceeded with using GENOMATIX prediction program. (C) Luciferase Activity of VASA promoter after NANOG OE and KD in cultured chicken PGCs (D) Western blot of VASA after NANOG KD and VPA treatment with DF1 for negative control

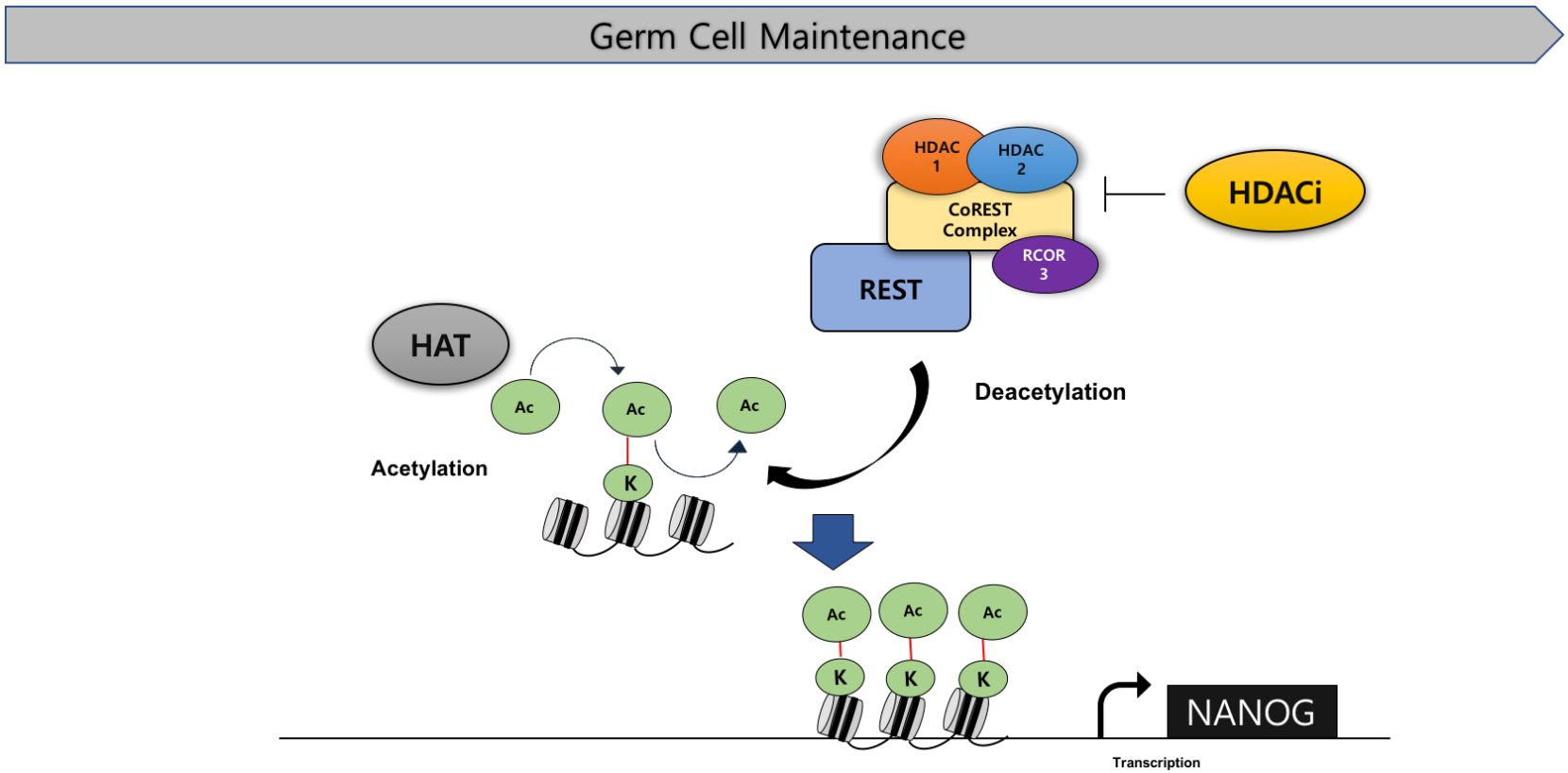


Figure 7. A Diagram of the role of epigenetic modifications in control of NANOG in chicken PGCs

Table 1. siRNA list of HDACs and their complexes

No.	Gene Symbol	Accession No.	Primer sequence (5' → 3')
1	REST	XM_015276447	5’-CAGAGACTGTCAGGAAGCTCAGAAA
			3-UUUCUGAGCUUCCUGACAGUCUCUG
2	SIN3A	NM_001293184	5’-ACAUCUUCAUGAGGCUGCAUCAGAU
			3’-AUCUGAUGCAGCCUCAUGAAGAUGU
3	SIN3B	NM_001293184	CAGCGGGCUGUUGUUGCAUUACUCA
			UGAGUAAUGCAACAACAGCCCGCUG
4	MTA1	NM_001012953	5’-CCGCCAAUGGAAAUGUGGAAGCAAA
			3’-UUUGCUUCCACAUUUCCAUUGGCGG
5	MTA3	XM_015283840	CAUGUACCGAGUGGGAGAUUAUGUU
			AACAUAAUCUCCCACUCGGUACAUG
6	RCOR1	XM_015287816	CCGACGUCGUUUCAACAUAGAUGAA
			UUCAUCUAUGUUGAAACGACGUCGG
7	RCOR3	NM_001079727	CGGAUCUCCCUAACUUCACUCCCUU
			AAGGGAGUGAAGUUAGGGAGAUCCG

Table 2. List of primers used for quantitative real-time PCR

No.	Gene Symbol	Description	Accession No.	Primer sequence (5' → 3')
1	REST	RE1-Silencing Transcription factor	XM_015276447	F: AAAGAGCAAACAAAAGGGGA
				R: TTGCTCGTTGGCTTCTTTT
2	SIN3A	SIN3 Transcription Regulator Family Member	NM_001293184	F: GGAAGAGGAGGAAGAAGAGG
				R: CTCTCCCTTGTCTCCTCTTC
3	SIN3B	RE1-Silencing Transcription factor	NM_001293184	F: GGGGAGAAAAGAAAAGACCAA
				R: CCTCGCTTCCTTTCGTTC
4	MTA1	Metastasis-associated protein 1	NM_001012953	F: AACAAGCCAAACCCCAACC
				R: GTTTGGTCCTGGTCTCTCTC
5	MTA3	metastasis associated 1 family, member 3	XM_015283840	F: GCTCTTCCTTTCTCGCCAGT
				R: ACGGTCTGTAAGGGGGCTAT
6	RCOR1	REST corepressor 1	XM_015287816	F: AACAGAGCGAAGAGGAAACC
				R: CTCTTTGCCGTGTTCTGC
7	RCOR3	REST corepressor 3	NM_001079727	F: ATCGACAGGCTCGAAAGCTTGCTA
				R: CATTCTCAGCGTTCCAAATGCCG

Table 3. List of primers used for chromatin immunoprecipitation (ChIP) - quantitative real-time PCR

			Primer sequences	
No.	Primer	Description	Forward (5'→3')	Reverse (5'→3')
1	ChIp-REST	Target site	CTCCCCCTCCATTCTTTGTACTTG	AAAAGTCAGTTGATTGGAGGGGAG
2	ChIp-non	Non - target site	TCGGAAGACGGCGCCATGCTATTT	ACCTGCAGGACCAGGAGCACGTTA

Table 4. NANOG gene (Accession No. NM_001146142.1) overexpression vector and siRNA designs

Type	Information
Overexpression backbone vector	pCE-hSK (Addgene; Plasmid #41814)
Overexpression vector insert : NANOG CDS	>NM_001146142.1 Gallus gallus Nanog homeobox (NANOG), mRNA ATGAGCGCTCACCTGGCCATGCCGTCCTACGGCTCTGTTAGGTGCGGACACTACTACTGGCCCTCT CGGGCAGCATGGATAGCGCGTCTGCCGCGGAAGCTCCAGCAGCAGACCTCTCCTTGACCACAGAG CAGAAAACGCCCTGCCACCCAGATGCCTCTCCAGCTTCTTCCAGCTCTGGGACACTCATTCAGTATA CCCCAGACTCTGCCACTAGCCCTACTGCAGACCACCCATCTCACCGCCCCACTTTTCAGAAGGTTAA GGATAAAGGTGAGAGTGGGACAAGGAAGGCCAAGAGCCGCACAGCTTTCTCCCAGGAGCAGCTG CAGACCCTGCACCAGCGGTTTCAGAGCCAGAAGTACCTCAGCCCCCATCAGATCCGGGAGCTGGCT GCTGCTCTGGGGCTCACCTACAAGCAGGTGAAGACGTGGTTTCAGAACCAACGAATGAAGTTTAAA CGTTGCCAGAAAGAGAGTCAGTGGGTGGATAAAGGGATTTATCTACCACAGAATGGGTTTCATCAA GCTGCGTATCTGGATATGACCCCTACATTTCACCAGGGCTTCCCTGTTGTTGCCAACAGAAACCTTC AGGCTGTGACCAGTGCACACCAGGCTTACAGCAGTGGCCAGACTTATGGGAATGGGCAGGGCCTG TATCCGTTCATGGCTGTGGAGGATGAGGGCTTCTTTGGAAAAGGTGGAACAAGCTGCAACACCCAG CAGGCCATGGGTTTATTAAGTCAACAGATGAACTTCTATCATGGCTACTCTACCAATGTGGATTATG ACAGCTTGCAGGCAGAAGATACCTACAGCTTCCAGAGCACCTCTGATAGTATCACACAGTTCTCGA GCTCTCCTGTACGGCATCAGTACCAGGCCCCTTGGCATACCCTGGGGACCCAGAATGGTTATGAGA CTTAG
Enzyme for insertion	ECoRI (G^AATTC)
siRNA - 836	Sense strand siRNA: CUGAUAGUAUCACACAGUU UU Antisense strand siRNA: AACUGUGUGAUACUAUCAG UU

Table 5. List of Antibodies

Product	Cat. No.	ISOTYPE	Reactivity	Applications
Anti-Histone H3 (acetyl K9) antibody	ab61231	Rabbit IgG	Mouse, Rat, Human	IHC-Fr, IHC-P, ICC/IF, WB
Anti-Histone H3 (acetyl K9) antibody	Upstate 06-942	Rabbit IgG	H, M, R	WB, ChIP, DB, FC, ChIP-seq
Anti-trimethyl-Histone H3 (Lys27) : H3K27me3	Millipore 07-449	Rabbit IgG	Human, mouse. Broad species cross-reactivity expected.	CC, IHC, ChIP-seq, WB, Mplex, IP
Anti-Histone H3 (tri methyl K9) antibody - ChIP Grade : H3K9me3	Upstate 07-442	Rabbit IgG	H, M, R, Ch	DB, ICC, Mplex, PIA, WB, ChIP-seq
Anti-Histone H3 (acetyl K27) antibody - ChIP Grade	ab4729	Rabbit IgG	Mouse, Rat, Chicken, Cow, Human, Arabidopsis thaliana, Drosophila melanogaster, Monkey, Zebrafish, Plasmodium falciparum, Rice, Cyanidioschyzon merolae	IHC-Fr, ICC/IF, WB, IHC-P, CHIPseq, ChIP/Chip, ChIP, PepArr

4. Discussion

In this study, we report that CoREST/HDAC can act as a transcriptional corepressor complex for regulation of germness in PGCs. Through ChIP-qPCR analyses and Immunocytochemistry, we identified that several pluripotency and germness genes that are directly upregulated, respectively, by NANOG and CoREST in PGCs.

It is worth pointing out that our findings necessarily match with those previous reports of chemicals on pluripotency gene regulation, though the HDAC complex between pluripotent stem cells and unipotent germ cells are different. In vertebrates, HDACs are determined to regulate gene expression to have a role in pluripotency or germ cell fate. Most of the studies in embryology were focused on specific HDAC complexes to regulate pluripotency in ESCs (Saunders, Huang et al. 2017). There are few studies in PGCs among species. In mouse, NuRD/HDAC complex regulated Sall4 repress Cdx2 (Yuri, Fujimura et al. 2009).

Thus, epigenetic studies of PGCs, such as HDAC need to be studied. In chicken, we found that CoREST/HDAC complex regulates NANOG in PGCs to regulate germ cell fate by transcriptional and translational pattern after knockdown and chemical treatment. And also, we identified the locus of REST binding site on cNANOG promoter to validate the regulation of NANOG by CoREST/HDAC Complex. Furthermore, we

also confirmed that VASA is regulated by NANOG in chicken PGCs, whether directly or indirectly unknown.

In this study, in contrast to our prediction, our result shows that both overexpression and knockdown of NANOG result in vanishing PGC integrity. In vertebrates, overexpression of NANOG causes tumor in germ cell (Hart, Hartley et al. 2005, Heaney, Anderson et al. 2012, Dovey, Foster et al. 2013). And also, knockdown of NANOG induces apoptotic cell death (Yamaguchi, Kurimoto et al. 2009, Dovey, Foster et al. 2013). To confirm that changes of gene expression pattern result in abnormal germ cell characteristics, tracing studies of NANOG pattern in germ cells using microinjection of overexpression vector or Cas9-derived knockout *in vivo* is acquired. And also, which stage in embryonic development the PGCs will be included after RA treatment is still ambiguous. Thus, for the further studies of the onset of meiosis (Smith, Roeszler et al. 2008), validation of the comparison that the exact stage RA treated PGCs are included will be needed.

REFERENCES

Acloque, H., O. H. Ocana, A. Matheu, K. Rizzoti, C. Wise, R. Lovell-Badge and M. A. Nieto (2011). "Reciprocal repression between Sox3 and snail transcription factors defines embryonic territories at gastrulation." Dev Cell **21**(3): 546-558.

Ara, T., Y. Nakamura, T. Egawa, T. Sugiyama, K. Abe, T. Kishimoto, Y. Matsui and T. Nagasawa (2003). "Impaired colonization of the gonads by primordial germ cells in mice lacking a chemokine, stromal cell-derived factor-1 (SDF-1)." Proc Natl Acad Sci U S A **100**(9): 5319-5323.

Arand, J., M. Wossidlo, K. Lepikhov, J. R. Peat, W. Reik and J. Walter (2015). "Selective impairment of methylation maintenance is the major cause of DNA methylation reprogramming in the early embryo." Epigenetics & chromatin **8**(1): 1.

Arkov, A. L. and A. Ramos (2010). "Building RNA-protein granules: insight from the germline." Trends Cell Biol **20**(8): 482-490.

Banerji, J., S. Rusconi and W. Schaffner (1981). "Expression of a beta-globin gene is enhanced by remote SV40 DNA sequences." Cell **27**(2 Pt 1): 299-308.

Barrios, A. P., A. V. Gomez, J. E. Saez, G. Ciossani, E. Toffolo, E. Battaglioli, A. Mattevi and M. E. Andres (2014). "Differential properties of transcriptional complexes formed by the CoREST family." Mol Cell Biol **34**(14): 2760-2770.

Bhullar, B., J. V. Schmidt, T. Truong, D. Rancourt and F. A. van der Hoorn (2001). "Germ cell specific promoter drives ectopic transgene expression during embryogenesis." Mol Reprod Dev **59**(1): 25-32.

Borgel, J., S. Guibert, Y. Li, H. Chiba, D. Schubeler, H. Sasaki, T. Forne and M. Weber (2010). "Targets and dynamics of promoter DNA methylation during early mouse development." Nat Genet **42**(12): 1093-1100.

Bucher, P., J. W. Fickett and A. Hatzigeorgiou (1996). "Computational analysis of

transcriptional regulatory elements: a field in flux." Comput Appl Biosci **12**(5): 361-362.

Butler, J. E. and J. T. Kadonaga (2002). "The RNA polymerase II core promoter: a key component in the regulation of gene expression." Genes Dev **16**(20): 2583-2592.

Canon, S., C. Herranz and M. Manzanares (2006). "Germ cell restricted expression of chick Nanog." Dev Dyn **235**(10): 2889-2894.

Carter, A. C., B. N. Davis-Dusenbery, K. Koszka, J. K. Ichida and K. Eggan (2014). "Nanog-independent reprogramming to iPSCs with canonical factors." Stem Cell Reports **2**(2): 119-126.

Chai, W., C. T. Yuen, T. Feizi and A. M. Lawson (1999). "Core-branching pattern and sequence analysis of mannitol-terminating oligosaccharides by neoglycolipid technology." Anal Biochem **270**(2): 314-322.

Chambers, I., D. Colby, M. Robertson, J. Nichols, S. Lee, S. Tweedie and A. Smith (2003). "Functional expression cloning of Nanog, a pluripotency sustaining factor in embryonic stem cells." Cell **113**(5): 643-655.

Chambers, I., J. Silva, D. Colby, J. Nichols, B. Nijmeijer, M. Robertson, J. Vrana, K. Jones, L. Grotewold and A. Smith (2007). "Nanog safeguards pluripotency and mediates germline development." Nature **450**(7173): 1230-1234.

Chan, K. K., J. Zhang, N. Y. Chia, Y. S. Chan, H. S. Sim, K. S. Tan, S. K. Oh, H. H. Ng and A. B. Choo (2009). "KLF4 and PBX1 directly regulate NANOG expression in human embryonic stem cells." Stem Cells **27**(9): 2114-2125.

Chang, P., J. Torres, R. A. Lewis, K. L. Mowry, E. Houliston and M. L. King (2004). "Localization of RNAs to the mitochondrial cloud in Xenopus oocytes through entrapment and association with endoplasmic reticulum." Mol Biol Cell **15**(10): 4669-4681.

Chapman, S. C., J. Collignon, G. C. Schoenwolf and A. Lumsden (2001). "Improved method for chick whole-embryo culture using a filter paper carrier." Dev Dyn **220**(3): 284-289.

Chen, Z. F., A. J. Paquette and D. J. Anderson (1998). "NRSF/REST is required in vivo for repression of multiple neuronal target genes during embryogenesis." Nat Genet **20**(2): 136-142.

Choi, J. W., S. Kim, T. M. Kim, Y. M. Kim, H. W. Seo, T. S. Park, J. W. Jeong, G. Song and J. Y. Han (2010). "Basic fibroblast growth factor activates MEK/ERK cell signaling pathway and stimulates the proliferation of chicken primordial germ cells." PLoS One **5**(9): e12968.

Chong, J. A., J. Tapia-Ramirez, S. Kim, J. J. Toledo-Aral, Y. Zheng, M. C. Boutros, Y. M. Altshuller, M. A. Frohman, S. D. Kraner and G. Mandel (1995). "REST: a mammalian silencer protein that restricts sodium channel gene expression to neurons." Cell **80**(6): 949-957.

Collier, B., B. Gorgoni, C. Loveridge, H. J. Cooke and N. K. Gray (2005). "The DAZL family proteins are PABP-binding proteins that regulate translation in germ cells." EMBO J **24**(14): 2656-2666.

Cowger, J. J., Q. Zhao, M. Isovic and J. Torchia (2007). "Biochemical characterization of the zinc-finger protein 217 transcriptional repressor complex: identification of a ZNF217 consensus recognition sequence." Oncogene **26**(23): 3378-3386.

Dallman, J. E., J. Allopenna, A. Bassett, A. Travers and G. Mandel (2004). "A conserved role but different partners for the transcriptional corepressor CoREST in fly and mammalian nervous system formation." J Neurosci **24**(32): 7186-7193.

De Felici, M. and F. Barrios (2013). "Seeking the origin of female germline stem cells in the mammalian ovary." Reproduction **146**(4): R125-R130.

de Ruijter, A. J., A. H. van Gennip, H. N. Caron, S. Kemp and A. B. van Kuilenburg

(2003). "Histone deacetylases (HDACs): characterization of the classical HDAC family." Biochem J **370**(Pt 3): 737-749.

de Sousa Lopes, S. M., K. Hayashi and M. A. Surani (2007). "Proximal visceral endoderm and extraembryonic ectoderm regulate the formation of primordial germ cell precursors." BMC Dev Biol **7**: 140.

Deppe, U., E. Schierenberg, T. Cole, C. Krieg, D. Schmitt, B. Yoder and G. von Ehrenstein (1978). "Cell lineages of the embryo of the nematode Caenorhabditis elegans." Proc Natl Acad Sci U S A **75**(1): 376-380.

Dolci, S., D. E. Williams, M. K. Ernst, J. L. Resnick, C. I. Brannan, L. F. Lock, S. D. Lyman, H. S. Boswell and P. J. Donovan (1991). "Requirement for mast cell growth factor for primordial germ cell survival in culture." Nature **352**(6338): 809-811.

Dovey, O. M., C. T. Foster, N. Conte, S. A. Edwards, J. M. Edwards, R. Singh, G. Vassiliou, A. Bradley and S. M. Cowley (2013). "Histone deacetylase 1 and 2 are essential for normal T-cell development and genomic stability in mice." Blood **121**(8): 1335-1344.

Dovey, O. M., C. T. Foster and S. M. Cowley (2010). "Histone deacetylase 1 (HDAC1), but not HDAC2, controls embryonic stem cell differentiation." Proc Natl Acad Sci U S A **107**(18): 8242-8247.

Durcova-Hills, G., P. Hajkova, S. Sullivan, S. Barton, M. A. Surani and A. McLaren (2006). "Influence of sex chromosome constitution on the genomic imprinting of germ cells." Proc Natl Acad Sci U S A **103**(30): 11184-11188.

Edson, M. A., Y. N. Lin and M. M. Matzuk (2010). "Deletion of the novel oocyte-enriched gene, Gpr149, leads to increased fertility in mice." Endocrinology **151**(1): 358-368.

Extavour, C. G. and M. Akam (2003). "Mechanisms of germ cell specification across the metazoans: epigenesis and preformation." Development

130(24): 5869-5884.

Eyalgiladi, H. and S. Kochav (1976). "From Cleavage to Primitive Streak Formation - Complementary Normal Table and a New Look at 1st Stages of Development of Chick .1. General Morphology." Developmental Biology **49**(2): 321-337.

Farini, D., M. L. Scaldaferri, S. Iona, G. La Sala and M. De Felici (2005). "Growth factors sustain primordial germ cell survival, proliferation and entering into meiosis in the absence of somatic cells." Dev Biol **285**(1): 49-56.

Fickett, J. W. and A. G. Hatzigeorgiou (1997). "Eukaryotic promoter recognition." Genome Res **7**(9): 861-878.

Fu, X. F., S. F. Cheng, L. Q. Wang, S. Yin, M. De Felici and W. Shen (2015). "DAZ Family Proteins, Key Players for Germ Cell Development." Int J Biol Sci **11**(10): 1226-1235.

Gardiner-Garden, M. and M. Frommer (1987). "CpG islands in vertebrate genomes." J Mol Biol **196**(2): 261-282.

Gong, D., W. Li, L. D. Hu, J. L. Shen, M. Y. Fang, Q. M. Yang, H. X. Wang, X. Y. Ke, H. R. Chen, Z. Wang, H. Liu, F. Liu, Y. G. Ma, J. W. Wang, H. H. Li, Q. S. Wang, Y. Jing, X. N. Gao, L. P. Dou, Y. H. Li, J. M. Luo and L. Yu (2016). "Comparison of Clinical Efficacy of Cytarabine with Different Regimens in Postremission Consolidation Therapy for Adult t(8;21) AML Patients: A Multicenter Retrospective Study in China." Acta Haematol **136**(4): 201-209.

Goodrich, J. A. and R. Tjian (2010). "Unexpected roles for core promoter recognition factors in cell-type-specific transcription and gene regulation." Nat Rev Genet **11**(8): 549-558.

Grimes, J. A., S. J. Nielsen, E. Battaglioli, E. A. Miska, J. C. Speh, D. L. Berry, F. Atouf, B. C. Holdener, G. Mandel and T. Kouzarides (2000). "The co-

repressor mSin3A is a functional component of the REST-CoREST repressor complex." J Biol Chem **275**(13): 9461-9467.

Grozinger, C. M. and S. L. Schreiber (2002). "Deacetylase enzymes: biological functions and the use of small-molecule inhibitors." Chem Biol **9**(1): 3-16.

Gu, T. P., F. Guo, H. Yang, H. P. Wu, G. F. Xu, W. Liu, Z. G. Xie, L. Shi, X. He, S. G. Jin, K. Iqbal, Y. G. Shi, Z. Deng, P. E. Szabo, G. P. Pfeifer, J. Li and G. L. Xu (2011). "The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes." Nature **477**(7366): 606-610.

Gu, Y., C. Runyan, A. Shoemaker, A. Surani and C. Wylie (2009). "Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration." Development **136**(8): 1295-1303.

Guibert, S., T. Forne and M. Weber (2012). "Global profiling of DNA methylation erasure in mouse primordial germ cells." Genome Res **22**(4): 633-641.

Guo, C., Y. Xue, G. Yang, S. Yin, W. Shi, Y. Cheng, X. Yan, S. Fan, H. Zhang and F. Zeng (2016). "Nanog RNA-binding proteins YBX1 and ILF3 affect pluripotency of embryonic stem cells." Cell Biol Int **40**(8): 847-860.

Gupta, R. A., N. Shah, K. C. Wang, J. Kim, H. M. Horlings, D. J. Wong, M. C. Tsai, T. Hung, P. Argani, J. L. Rinn, Y. Wang, P. Brzoska, B. Kong, R. Li, R. B. West, M. J. van de Vijver, S. Sukumar and H. Y. Chang (2010). "Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis." Nature **464**(7291): 1071-1076.

Gustafson, E. A. and G. M. Wessel (2010). "Vasa genes: emerging roles in the germ line and in multipotent cells." Bioessays **32**(7): 626-637.

Hammachi, F., G. M. Morrison, A. A. Sharov, A. Livigni, S. Narayan, E. P. Papapetrou, J. O'Malley, K. Kaji, M. S. Ko, M. Ptashne and J. M. Brickman (2012). "Transcriptional activation by Oct4 is sufficient for

the maintenance and induction of pluripotency." Cell Rep **1**(2): 99-109.

Han, J. Y., T. S. Park, J. N. Kim, M. A. Kim, D. Lim, J. M. Lim and H. Kim (2006). "Gene expression profiling of chicken primordial germ cell ESTs." BMC genomics **7**(1): 220.

Han, S., W. Xie, S. H. Kim, L. Yue and J. DeJong (2004). "A short core promoter drives expression of the ALF transcription factor in reproductive tissues of male and female mice." Biol Reprod **71**(3): 933-941.

Hart, A. H., L. Hartley, M. Ibrahim and L. Robb (2004). "Identification, cloning and expression analysis of the pluripotency promoting Nanog genes in mouse and human." Dev Dyn **230**(1): 187-198.

Hart, A. H., L. Hartley, K. Parker, M. Ibrahim, L. H. Looijenga, M. Pauchnik, C. W. Chow and L. Robb (2005). "The pluripotency homeobox gene NANOG is expressed in human germ cell tumors." Cancer **104**(10): 2092-2098.

Hayashi, K., H. Ohta, K. Kurimoto, S. Aramaki and M. Saitou (2011). "Reconstitution of the mouse germ cell specification pathway in culture by pluripotent stem cells." Cell **146**(4): 519-532.

Heaney, J. D., E. L. Anderson, M. V. Michelson, J. L. Zechel, P. A. Conrad, D. C. Page and J. H. Nadeau (2012). "Germ cell pluripotency, premature differentiation and susceptibility to testicular teratomas in mice." Development **139**(9): 1577-1586.

Hird, S. N., J. E. Paulsen and S. Strome (1996). "Segregation of germ granules in living Caenorhabditis elegans embryos: cell-type-specific mechanisms for cytoplasmic localisation." Development **122**(4): 1303-1312.

Houston, D. W. and M. L. King (2000). "Germ plasm and molecular determinants of germ cell fate." Curr Top Dev Biol **50**: 155-181.

Hyslop, L., M. Stojkovic, L. Armstrong, T. Walter, P. Stojkovic, S. Przyborski, M.

Herbert, A. Murdoch, T. Strachan and M. Lako (2005). "Downregulation of NANOG induces differentiation of human embryonic stem cells to extraembryonic lineages." Stem Cells **23**(8): 1035-1043.

Ikawa, M., K. Tokuhiro, R. Yamaguchi, A. M. Benham, T. Tamura, I. Wada, Y. Satouh, N. Inoue and M. Okabe (2011). "Calsperin is a testis-specific chaperone required for sperm fertility." J Biol Chem **286**(7): 5639-5646.

Illmensee, K. and A. P. Mahowald (1974). "Transplantation of posterior polar plasm in Drosophila. Induction of germ cells at the anterior pole of the egg." Proc Natl Acad Sci U S A **71**(4): 1016-1020.

Irie, N., L. Weinberger, W. W. Tang, T. Kobayashi, S. Viukov, Y. S. Manor, S. Dietmann, J. H. Hanna and M. A. Surani (2015). "SOX17 is a critical specifier of human primordial germ cell fate." Cell **160**(1-2): 253-268.

Jang, H. J., H. W. Seo, B. R. Lee, M. Yoo, J. E. Womack and J. Y. Han (2013). "Gene expression and DNA methylation status of chicken primordial germ cells." Mol Biotechnol **54**(2): 177-186.

Jauch, R., C. K. Ng, K. S. Saikatendu, R. C. Stevens and P. R. Kolatkar (2008). "Crystal structure and DNA binding of the homeodomain of the stem cell transcription factor Nanog." J Mol Biol **376**(3): 758-770.

Jean, C., N. M. Oliveira, S. Intarapat, A. Fuet, C. Mazoyer, I. De Almeida, K. Trevers, S. Boast, P. Aubel, F. Bertocchini, C. D. Stern and B. Pain (2015). "Transcriptome analysis of chicken ES, blastodermal and germ cells reveals that chick ES cells are equivalent to mouse ES cells rather than EpiSC." Stem Cell Res **14**(1): 54-67.

Jiao, F., X. Wang, Z. Yan, C. Liu, Z. Yue, Z. Li, Y. Ma, Y. Li and J. Wang (2013). "Effect of dynamic DNA methylation and histone acetylation on cPouV expression in differentiation of chick embryonic germ cells."

Stem Cells Dev **22**(20): 2725-2735.

Jimenez, R., E. O. Melo, O. Davydenko, J. Ma, M. Mainigi, V. Franke and R. M. Schultz (2015). "Maternal SIN3A regulates reprogramming of gene expression during mouse preimplantation development." Biol Reprod **93**(4): 89.

Jin, S. (2016). "Transcrptome Analyses and cis - Regulatory Element Assay of Germ Cell-Specific Genes in Chicken Primoirdial Germ Cells." Unpublished Masteral Dissertion, Seoul National University, Seoul, Korea.

Juven-Gershon, T. and J. T. Kadonaga (2010). "Regulation of gene expression via the core promoter and the basal transcriptional machinery." Dev Biol **339**(2): 225-229.

Kadonaga, J. T. (2012). "Perspectives on the RNA polymerase II core promoter." Wiley Interdiscip Rev Dev Biol **1**(1): 40-51.

Kadosh, D. and K. Struhl (1997). "Repression by Ume6 involves recruitment of a complex containing Sin3 corepressor and Rpd3 histone deacetylase to target promoters." Cell **89**(3): 365-371.

Kanai-Azuma, M., Y. Kanai, J. M. Gad, Y. Tajima, C. Taya, M. Kurohmaru, Y. Sanai, H. Yonekawa, K. Yazaki, P. P. Tam and Y. Hayashi (2002). "Depletion of definitive gut endoderm in Sox17-null mutant mice." Development **129**(10): 2367-2379.

Kangaspeska, S., B. Stride, R. Metivier, M. Polycarpou-Schwarz, D. Ibberson, R. P. Carmouche, V. Benes, F. Gannon and G. Reid (2008). "Transient cyclical methylation of promoter DNA." Nature **452**(7183): 112-115.

Kawasaki, Y., J. Lee, A. Matsuzawa, T. Kohda, T. Kaneko-Ishino and F. Ishino (2014). "Active DNA demethylation is required for complete imprint erasure in primordial germ cells." Scientific Reports **4**(1).

Kehler, J., E. Tolkunova, B. Koschorz, M. Pesce, L. Gentile, M. Boiani, H. Lomeli, A.

Nagy, K. J. McLaughlin, H. R. Scholer and A. Tomilin (2004). "Oct4 is required for primordial germ cell survival." EMBO Rep **5**(11): 1078-1083.

Kelly, R. D. and S. M. Cowley (2013). "The physiological roles of histone deacetylase (HDAC) 1 and 2: complex co-stars with multiple leading parts." Biochem Soc Trans **41**(3): 741-749.

Khromov, T., D. V. Pantakani, J. Nolte, M. Wolf, R. Dressel, W. Engel and U. Zechner (2011). "Global and gene-specific histone modification profiles of mouse multipotent adult germline stem cells." Mol Hum Reprod **17**(3): 166-174.

Kim, H., T. S. Park, W. K. Lee, S. Moon, J. N. Kim, J. H. Shin, J. G. Jung, S. D. Lee, S. H. Park and K. J. Park (2007). "MPSS profiling of embryonic gonad and primordial germ cells in chicken." Physiological genomics **29**(3): 253-259.

Kim, J. M., H. Liu, M. Tazaki, M. Nagata and F. Aoki (2003). "Changes in histone acetylation during mouse oocyte meiosis." J Cell Biol **162**(1): 37-46.

Kim, M., D. Li, Y. Cui, K. Mueller, W. C. Chears and J. DeJong (2006). "Regulatory factor interactions and somatic silencing of the germ cell-specific ALF gene." J Biol Chem **281**(45): 34288-34298.

Kress, C., G. Montillet, C. Jean, A. Fuet and B. Pain (2016). "Chicken embryonic stem cells and primordial germ cells display different heterochromatic histone marks than their mammalian counterparts." Epigenetics Chromatin **9**: 5.

Kunwar, P. S., H. Sano, A. D. Renault, V. Barbosa, N. Fuse and R. Lehmann (2008). "Tre1 GPCR initiates germ cell transepithelial migration by regulating Drosophila melanogaster E-cadherin." J Cell Biol **183**(1): 157-168.

Kurimoto, K., Y. Yabuta, K. Hayashi, H. Ohta, H. Kiyonari, T. Mitani, Y. Moritoki, K. Kohri, H. Kimura and T. Yamamoto (2015). "Quantitative dynamics of

chromatin remodeling during germ cell specification from mouse embryonic stem cells." Cell stem cell **16**(5): 517-532.

Kuroda, T., M. Tada, H. Kubota, H. Kimura, S. Y. Hatano, H. Suemori, N. Nakatsuji and T. Tada (2005). "Octamer and Sox elements are required for transcriptional cis regulation of Nanog gene expression." Mol Cell Biol **25**(6): 2475-2485.

Lasko, P. F. and M. Ashburner (1988). "The product of the Drosophila gene vasa is very similar to eukaryotic initiation factor-4A." Nature **335**(6191): 611-617.

Lavial, F., H. Acloque, E. Bachelard, M. A. Nieto, J. Samarut and B. Pain (2009). "Ectopic expression of Cvh (Chicken Vasa homologue) mediates the reprogramming of chicken embryonic stem cells to a germ cell fate." Dev Biol **330**(1): 73-82.

Lee, H. C., H. J. Choi, H. G. Lee, J. M. Lim, T. Ono and J. Y. Han (2016). "DAZL Expression Explains Origin and Central Formation of Primordial Germ Cells in Chickens." Stem Cells and Development **25**(1): 68-79.

Lee, S. H., T. Y. Chen, S. S. Dhar, B. Gu, K. Chen, Y. Z. Kim, W. Li and M. G. Lee (2016). "A feedback loop comprising PRMT7 and miR-24-2 interplays with Oct4, Nanog, Klf4 and c-Myc to regulate stemness." Nucleic Acids Res **44**(22): 10603-10618.

Lee, S. I., B. R. Lee, Y. S. Hwang, H. C. Lee, D. Rengaraj, G. Song, T. S. Park and J. Y. Han (2011). "MicroRNA-mediated posttranscriptional regulation is required for maintaining undifferentiated properties of blastoderm and primordial germ cells in chickens." Proceedings of the National Academy of Sciences **108**(26): 10426-10431.

Leitch, H. G. and A. Smith (2013). "The mammalian germline as a pluripotency cycle." Development **140**(12): 2495-2501.

Lenhard, B., A. Sandelin and P. Carninci (2012). "Metazoan promoters: emerging

characteristics and insights into transcriptional regulation." Nat Rev Genet **13**(4): 233-245.

Lesch, B. J. and D. C. Page (2012). "Genetics of germ cell development." Nat Rev Genet **13**(11): 781-794.

Lin, Y. and D. C. Page (2005). "Dazl deficiency leads to embryonic arrest of germ cell development in XY C57BL/6 mice." Dev Biol **288**(2): 309-316.

Lister, R., E. A. Mukamel, J. R. Nery, M. Urich, C. A. Puddifoot, N. D. Johnson, J. Lucero, Y. Huang, A. J. Dwork, M. D. Schultz, M. Yu, J. Tonti-Filippini, H. Heyn, S. Hu, J. C. Wu, A. Rao, M. Esteller, C. He, F. G. Haghighi, T. J. Sejnowski, M. M. Behrens and J. R. Ecker (2013). "Global epigenomic reconfiguration during mammalian brain development." Science **341**(6146): 1237905.

Ma, P., E. de Waal, J. R. Weaver, M. S. Bartolomei and R. M. Schultz (2015). "A DNMT3A2-HDAC2 Complex Is Essential for Genomic Imprinting and Genome Integrity in Mouse Oocytes." Cell Rep **13**(8): 1552-1560.

Macdonald, J., J. D. Glover, L. Taylor, H. M. Sang and M. J. McGrew (2010). "Characterisation and germline transmission of cultured avian primordial germ cells." PLoS One **5**(11): e15518.

Magnusdottir, E., S. Dietmann, K. Murakami, U. Gunesdogan, F. Tang, S. Bao, E. Diamanti, K. Lao, B. Gottgens and M. Azim Surani (2013). "A tripartite transcription factor network regulates primordial germ cell specification in mice." Nat Cell Biol **15**(8): 905-915.

Mahowald, A. P. (2001). "Assembly of the Drosophila germ plasm." Int Rev Cytol **203**: 187-213.

Matharu, N. and N. Ahituv (2015). "Minor Loops in Major Folds: Enhancer-Promoter Looping, Chromatin Restructuring, and Their Association with Transcriptional Regulation and Disease." PLoS Genet **11**(12): e1005640.

Matsui, Y., K. M. Zsebo and B. L. Hogan (1990). "Embryonic expression of a haematopoietic growth factor encoded by the Sl locus and the ligand for c-kit." Nature **347**(6294): 667-669.

Mitsui, K., Y. Tokuzawa, H. Itoh, K. Segawa, M. Murakami, K. Takahashi, M. Maruyama, M. Maeda and S. Yamanaka (2003). "The homeoprotein Nanog is required for maintenance of pluripotency in mouse epiblast and ES cells." Cell **113**(5): 631-642.

Molyneaux, K. A., H. Zinszner, P. S. Kunwar, K. Schaible, J. Stebler, M. J. Sunshine, W. O'Brien, E. Raz, D. Littman, C. Wylie and R. Lehmann (2003). "The chemokine SDF1/CXCL12 and its receptor CXCR4 regulate mouse germ cell migration and survival." Development **130**(18): 4279-4286.

Murakami, K., U. Gunesdogan, J. J. Zylicz, W. W. Tang, R. Sengupta, T. Kobayashi, S. Kim, R. Butler, S. Dietmann and M. A. Surani (2016). "NANOG alone induces germ cells in primed epiblast in vitro by activation of enhancers." Nature **529**(7586): 403-407.

Nakaki, F. and M. Saitou (2014). "PRDM14: a unique regulator for pluripotency and epigenetic reprogramming." Trends Biochem Sci **39**(6): 289-298.

Ng, J. H., V. Kumar, M. Muratani, P. Kraus, J. C. Yeo, L. P. Yaw, K. Xue, T. Lufkin, S. Prabhakar and H. H. Ng (2013). "In vivo epigenomic profiling of germ cells reveals germ cell molecular signatures." Dev Cell **24**(3): 324-333.

Novina, C. D. and A. L. Roy (1996). "Core promoters and transcriptional control." Trends Genet **12**(9): 351-355.

Novo, C. L., C. Tang, K. Ahmed, U. Djuric, E. Fussner, N. P. Mullin, N. P. Morgan, J. Hayre, A. R. Sienerth, S. Elderkin, R. Nishinakamura, I. Chambers, J. Ellis, D. P. Bazett-Jones and P. J. Rugg-Gunn (2016). "The pluripotency factor Nanog regulates pericentromeric heterochromatin organization in mouse embryonic stem cells." Genes Dev **30**(9): 1101-1115.

Ohinata, Y., H. Ohta, M. Shigeta, K. Yamanaka, T. Wakayama and M. Saitou

(2009). "A signaling principle for the specification of the germ cell lineage in mice." Cell **137**(3): 571-584.

Pain, B., M. E. Clark, M. Shen, H. Nakazawa, M. Sakurai, J. Samarut and R. J. Etches (1996). "Long-term in vitro culture and characterisation of avian embryonic stem cells with multiple morphogenetic potentialities." Development **122**(8): 2339-2348.

Pan, G. and J. A. Thomson (2007). "Nanog and transcriptional networks in embryonic stem cell pluripotency." Cell Res **17**(1): 42-49.

Pannett, C. and A. Compton (1924). "The cultivation of tissues in saline embryonic juice." The Lancet **203**(5243): 381-384.

Park, T. S. and J. Y. Han (2012). "Genetic modification of chicken germ cells." Ann N Y Acad Sci **1271**: 104-109.

Park, T. S. and J. Y. Han (2012). "piggyBac transposition into primordial germ cells is an efficient tool for transgenesis in chickens." Proc Natl Acad Sci U S A **109**(24): 9337-9341.

Pokholok, D. K., C. T. Harbison, S. Levine, M. Cole, N. M. Hannett, T. I. Lee, G. W. Bell, K. Walker, P. A. Rolfe, E. Herbolsheimer, J. Zeitlinger, F. Lewitter, D. K. Gifford and R. A. Young (2005). "Genome-wide map of nucleosome acetylation and methylation in yeast." Cell **122**(4): 517-527.

Reik, W. (2007). "Stability and flexibility of epigenetic gene regulation in mammalian development." Nature **447**(7143): 425-432.

Rengaraj, D., B. R. Lee, J. W. Choi, S. I. Lee, H. W. Seo, T. H. Kim, H. J. Choi, G. Song and J. Y. Han (2012). "Gene pathways and cell cycle-related genes in cultured avian primordial germ cells." Poult Sci **91**(12): 3167-3177.

Rengaraj, D., Y. H. Zheng, K. S. Kang, K. J. Park, B. R. Lee, S. I. Lee, J. W. Choi and J. Y. Han (2010). "Conserved expression pattern of chicken DAZL in primordial germ cells and germ-line cells." Theriogenology **74**(5):

765-776.

Ridsdale, J. A., M. J. Hendzel, G. P. Delcuve and J. R. Davie (1990). "Histone acetylation alters the capacity of the H1 histones to condense transcriptionally active/competent chromatin." J Biol Chem **265**(9): 5150-5156.

Ripamonti, U., C. Ferretti, J. Teare and L. Blann (2009). "Transforming growth factor-beta isoforms and the induction of bone formation: implications for reconstructive craniofacial surgery." J Craniofac Surg **20**(5): 1544-1555.

Robertson, J. C., N. C. Hurley, M. Tortorici, G. Ciossani, M. T. Borrello, N. A. Vellore, A. Ganesan, A. Mattevi and R. Baron (2013). "Expanding the druggable space of the LSD1/CoREST epigenetic target: new potential binding regions for drug-like molecules, peptides, protein partners, and chromatin." PLoS Comput Biol **9**(7): e1003158.

Rodda, D. J., J. L. Chew, L. H. Lim, Y. H. Loh, B. Wang, H. H. Ng and P. Robson (2005). "Transcriptional regulation of nanog by OCT4 and SOX2." J Biol Chem **280**(26): 24731-24737.

Rothbart, S. B. and B. D. Strahl (2014). "Interpreting the language of histone and DNA modifications." Biochim Biophys Acta **1839**(8): 627-643.

Ruggiu, M., R. Speed, M. Taggart, S. J. McKay, F. Kilanowski, P. Saunders, J. Dorin and H. J. Cooke (1997). "The mouse Dazla gene encodes a cytoplasmic protein essential for gametogenesis." Nature **389**(6646): 73-77.

Ruijtenberg, S. and S. van den Heuvel (2016). "Coordinating cell proliferation and differentiation: Antagonism between cell cycle regulators and cell type-specific gene expression." Cell Cycle **15**(2): 196-212.

Saito, H., K. Takeda, K. Yasumoto, H. Ohtani, K. Watanabe, K. Takahashi, A. Fukuzaki, Y. Arai, H. Yamamoto and S. Shibahara (2003). "Germ cell-

specific expression of microphthalmia-associated transcription factor mRNA in mouse testis." J Biochem **134**(1): 143-150.

Saitou, M. and M. Yamaji (2010). "Germ cell specification in mice: signaling, transcription regulation, and epigenetic consequences." Reproduction **139**(6): 931-942.

Sarmah, S., P. Muralidharan, C. L. Curtis, J. N. McClintick, B. B. Buente, D. J. Holdgrafer, O. Ogbeifun, O. C. Olorungbounmi, L. Patino, R. Lucas, S. Gilbert, E. S. Groninger, J. Arciero, H. J. Edenberg and J. A. Marrs (2013). "Ethanol exposure disrupts extraembryonic microtubule cytoskeleton and embryonic blastomere cell adhesion, producing epiboly and gastrulation defects." Biol Open **2**(10): 1013-1021.

Sasaki, H. and Y. Matsui (2008). "Epigenetic events in mammalian germ-cell development: reprogramming and beyond." Nat Rev Genet **9**(2): 129-140.

Saunders, A., F. Faiola and J. Wang (2013). "Concise review: pursuing self-renewal and pluripotency with the stem cell factor Nanog." Stem Cells **31**(7): 1227-1236.

Saunders, A., X. Huang, M. Fidalgo, M. H. Reimer, F. Faiola, J. Ding, C. Sánchez-Priego, D. Guallar, C. Sáenz and D. Li (2017). "The SIN3A/HDAC corepressor complex functionally cooperates with NANOG to promote pluripotency." Cell reports **18**(7): 1713-1726.

Saunders, A., X. Huang, M. Fidalgo, M. H. Reimer, Jr., F. Faiola, J. Ding, C. Sanchez-Priego, D. Guallar, C. Saenz, D. Li and J. Wang (2017). "The SIN3A/HDAC Corepressor Complex Functionally Cooperates with NANOG to Promote Pluripotency." Cell Rep **18**(7): 1713-1726.

Schug, J., W. P. Schuller, C. Kappen, J. M. Salbaum, M. Bucan and C. J. Stoeckert, Jr. (2005). "Promoter features related to tissue specificity as measured by Shannon entropy." Genome Biol **6**(4): R33.

Schulz, W. A. and M. J. Hoffmann (2007). "Transcription factor networks in embryonic stem cells and testicular cancer and the definition of epigenetics." Epigenetics **2**(1): 37-42.

Seisenberger, S., J. R. Peat, T. A. Hore, F. Santos, W. Dean and W. Reik (2013). "Reprogramming DNA methylation in the mammalian life cycle: building and breaking epigenetic barriers." Philos Trans R Soc Lond B Biol Sci **368**(1609): 20110330.

Seki, Y., K. Hayashi, K. Itoh, M. Mizugaki, M. Saitou and Y. Matsui (2005). "Extensive and orderly reprogramming of genome-wide chromatin modifications associated with specification and early development of germ cells in mice." Dev Biol **278**(2): 440-458.

Sekinaka, T., Y. Hayashi, T. Noce, H. Niwa and Y. Matsui (2016). "Selective de-repression of germ cell-specific genes in mouse embryonic fibroblasts in a permissive epigenetic environment." Sci Rep **6**: 32932.

Shahbazian, M. D. and M. Grunstein (2007). "Functions of site-specific histone acetylation and deacetylation." Annu Rev Biochem **76**: 75-100.

Shi, W., H. Wang, G. Pan, Y. Geng, Y. Guo and D. Pei (2006). "Regulation of the pluripotency marker Rex-1 by Nanog and Sox2." J Biol Chem **281**(33): 23319-23325.

Shi, Y. and J. R. Whetstine (2007). "Dynamic regulation of histone lysine methylation by demethylases." Mol Cell **25**(1): 1-14.

Singh, A. M., T. Hamazaki, K. E. Hankowski and N. Terada (2007). "A heterogeneous expression pattern for Nanog in embryonic stem cells." Stem Cells **25**(10): 2534-2542.

Smallwood, S. A. and G. Kelsey (2012). "De novo DNA methylation: a germ cell perspective." Trends Genet **28**(1): 33-42.

Smialowska, A. and R. Baumeister (2006). "Presenilin function in Caenorhabditis elegans." Neurodegener Dis **3**(4-5): 227-232.

Smith, C. A., K. N. Roeszler, J. Bowles, P. Koopman and A. H. Sinclair (2008). "Onset of meiosis in the chicken embryo; evidence of a role for retinoic acid." BMC developmental biology **8**(1): 85.

Smith, Z. D., M. M. Chan, T. S. Mikkelsen, H. Gu, A. Gnirke, A. Regev and A. Meissner (2012). "A unique regulatory phase of DNA methylation in the early mammalian embryo." Nature **484**(7394): 339-344.

Song, Y., L. Lai, L. Li, Y. Huang, A. Wang, X. Tang, D. Pang, Z. Li and H. Ouyang (2016). "Germ cell-specific expression of Cre recombinase using the VASA promoter in the pig." FEBS Open Bio **6**(1): 50-55.

Spitz, F. and E. E. Furlong (2012). "Transcription factors: from enhancer binding to developmental control." Nature Reviews Genetics **13**(9): 613-626.

Stadler, M. B., R. Murr, L. Burger, R. Ivanek, F. Lienert, A. Scholer, E. van Nimwegen, C. Wirbelauer, E. J. Oakeley, D. Gaidatzis, V. K. Tiwari and D. Schubeler (2011). "DNA-binding factors shape the mouse methylome at distal regulatory regions." Nature **480**(7378): 490-495.

Stebler, J., D. Spieler, K. Slanchev, K. A. Molyneaux, U. Richter, V. Cojocaru, V. Tarabykin, C. Wylie, M. Kessel and E. Raz (2004). "Primordial germ cell migration in the chick and mouse embryo: the role of the chemokine SDF-1/CXCL12." Dev Biol **272**(2): 351-361.

Strome, S. and D. Updike (2015). "Specifying and protecting germ cell fate." Nat Rev Mol Cell Biol **16**(7): 406-416.

Strome, S. and W. B. Wood (1982). "Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of Caenorhabditis elegans." Proc Natl Acad Sci U S A **79**(5): 1558-1562.

Su, X., S. Kameoka, S. Lentz and S. Majumder (2004). "Activation of REST/NRSF target genes in neural stem cells is sufficient to cause neuronal differentiation." Mol Cell Biol **24**(18): 8018-8025.

Swift, C. H. (1914). "Origin and early history of the primordial germ-cells in the

chick." American Journal of Anatomy **15**(4): 483-516.

Tada, H., M. Mochii, H. Orii and K. Watanabe (2012). "Ectopic formation of primordial germ cells by transplantation of the germ plasm: direct evidence for germ cell determinant in Xenopus." Dev Biol **371**(1): 86-93.

Takami, Y., H. Kikuchi and T. Nakayama (1999). "Chicken histone deacetylase-2 controls the amount of the IgM H-chain at the steps of both transcription of its gene and alternative processing of its pre-mRNA in the DT40 cell line." J Biol Chem **274**(34): 23977-23990.

Takeda, Y., Y. Mishima, T. Fujiwara, H. Sakamoto and K. Inoue (2009). "DAZL relieves miRNA-mediated repression of germline mRNAs by controlling poly(A) tail length in zebrafish." PLoS One **4**(10): e7513.

TANAKA, H., S. Tomoki, N. YAMAUCHI and M.-a. HATTORI (2014). "The Putative Promoters of Germ Cell-specific Genes and Nanog are Hypomethylated in Chicken Sperm." Journal of Reproduction and Development **60**(3): 224-229.

Tang, W. W., S. Dietmann, N. Irie, H. G. Leitch, V. I. Floros, C. R. Bradshaw, J. A. Hackett, P. F. Chinnery and M. A. Surani (2015). "A Unique Gene Regulatory Network Resets the Human Germline Epigenome for Development." Cell **161**(6): 1453-1467.

Tang, W. W., T. Kobayashi, N. Irie, S. Dietmann and M. A. Surani (2016). "Specification and epigenetic programming of the human germ line." Nat Rev Genet **17**(10): 585-600.

Tang, X. and C. Zhang (2007). "Activation of protein kinases A and C promoted proliferation of chicken primordial germ cells." Anim Reprod Sci **101**(3-4): 295-303.

Tollervey, J. R. and V. V. Lunyak (2012). "Epigenetics: judge, jury and executioner of stem cell fate." Epigenetics **7**(8): 823-840.

Torres, J. and F. M. Watt (2008). "Nanog maintains pluripotency of mouse embryonic stem cells by inhibiting NFkappaB and cooperating with Stat3." Nat Cell Biol **10**(2): 194-201.

Toyooka, Y., N. Tsunekawa, Y. Takahashi, Y. Matsui, M. Satoh and T. Noce (2000). "Expression and intracellular localization of mouse Vasa-homologue protein during germ cell development." Mech Dev **93**(1-2): 139-149.

Tsai, M. C., O. Manor, Y. Wan, N. Mosammaparast, J. K. Wang, F. Lan, Y. Shi, E. Segal and H. Y. Chang (2010). "Long noncoding RNA as modular scaffold of histone modification complexes." Science **329**(5992): 689-693.

Tsunekawa, N., M. Naito, Y. Sakai, T. Nishida and T. Noce (2000). "Isolation of chicken vasa homolog gene and tracing the origin of primordial germ cells." Development **127**(12): 2741-2750.

Tu, S., V. Narendra, M. Yamaji, S. E. Vidal, L. A. Rojas, X. Wang, S. Y. Kim, B. A. Garcia, T. Tuschl, M. Stadtfeld and D. Reinberg (2016). "Co-repressor CBFA2T2 regulates pluripotency and germline development." Nature **534**(7607): 387-390.

van de Lavoir, M. C., J. H. Diamond, P. A. Leighton, C. Mather-Love, B. S. Heyer, R. Bradshaw, A. Kerchner, L. T. Hooi, T. M. Gessaro, S. E. Swanberg, M. E. Delany and R. J. Etches (2006). "Germline transmission of genetically modified primordial germ cells." Nature **441**(7094): 766-769.

Walter, M., A. Teissandier, R. Pérez-Palacios and D. Bourc'his (2016). "An epigenetic switch ensures transposon repression upon dynamic loss of DNA methylation in embryonic stem cells." Elife **5**: e11418.

Wang, C., J. E. Lee, B. Lai, T. S. Macfarlan, S. Xu, L. Zhuang, C. Liu, W. Peng and K. Ge (2016). "Enhancer priming by H3K4 methyltransferase MLL4 controls cell fate transition." Proc Natl Acad Sci U S A **113**(42): 11871-11876.

Wang, H., Y. Liu, D. Ye, J. Li, J. Liu and F. Deng (2016). "Knockdown of zebrafish Nanog increases primordial germ cells during early embryonic development." Dev Growth Differ **58**(4): 355-366.

Wang, X., Y. Wang, Q. Zuo, D. Li, W. Zhang, C. Lian, B. Tang, T. Xiao, M. Wang, K. Wang, B. Li and Y. Zhang (2016). "The synergistic effect of 5Azadc and TSA on maintenance of pluripotency of chicken ESCs by overexpression of NANOG gene." In Vitro Cell Dev Biol Anim **52**(4): 488-496.

Wang, Z., C. Zang, K. Cui, D. E. Schones, A. Barski, W. Peng and K. Zhao (2009). "Genome-wide mapping of HATs and HDACs reveals distinct functions in active and inactive genes." Cell **138**(5): 1019-1031.

Weidinger, G., J. Stebler, K. Slanchev, K. Dumstrei, C. Wise, R. Lovell-Badge, C. Thisse, B. Thisse and E. Raz (2003). "dead end, a novel vertebrate germ plasm component, is required for zebrafish primordial germ cell migration and survival." Curr Biol **13**(16): 1429-1434.

White-Cooper, H. and I. Davidson (2011). "Unique aspects of transcription regulation in male germ cells." Cold Spring Harb Perspect Biol **3**(7).

Whyte, J., J. D. Glover, M. Woodcock, J. Brzeszczynska, L. Taylor, A. Sherman, P. Kaiser and M. J. McGrew (2015). "FGF, Insulin, and SMAD Signaling Cooperate for Avian Primordial Germ Cell Self-Renewal." Stem Cell Reports **5**(6): 1171-1182.

Wiren, M., R. A. Silverstein, I. Sinha, J. Walfridsson, H. M. Lee, P. Laurenson, L. Pillus, D. Robyr, M. Grunstein and K. Ekwall (2005). "Genomewide analysis of nucleosome density histone acetylation and HDAC function in fission yeast." EMBO J **24**(16): 2906-2918.

Wossidlo, M., T. Nakamura, K. Lepikhov, C. J. Marques, V. Zakhartchenko, M. Boiani, J. Arand, T. Nakano, W. Reik and J. Walter (2011). "5-Hydroxymethylcytosine in the mammalian zygote is linked with

epigenetic reprogramming." Nat Commun **2**: 241.

Xiao, L., M. Kim and J. DeJong (2006). "Developmental and cell type-specific regulation of core promoter transcription factors in germ cells of frogs and mice." Gene Expr Patterns **6**(4): 409-419.

Yamaguchi, S., H. Kimura, M. Tada, N. Nakatsuji and T. Tada (2005). "Nanog expression in mouse germ cell development." Gene Expr Patterns **5**(5): 639-646.

Yamaguchi, S., K. Kurimoto, Y. Yabuta, H. Sasaki, N. Nakatsuji, M. Saitou and T. Tada (2009). "Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells." Development **136**(23): 4011-4020.

Yamaguchi, S., K. Kurimoto, Y. Yabuta, H. Sasaki, N. Nakatsuji, M. Saitou and T. Tada (2009). "Conditional knockdown of Nanog induces apoptotic cell death in mouse migrating primordial germ cells." Development **136**(23): 4011-4020.

Yamaguchi, T., F. Cubizolles, Y. Zhang, N. Reichert, H. Kohler, C. Seiser and P. Matthias (2010). "Histone deacetylases 1 and 2 act in concert to promote the G1-to-S progression." Genes Dev **24**(5): 455-469.

Yan, C. and D. D. Boyd (2006). "Histone H3 acetylation and H3 K4 methylation define distinct chromatin regions permissive for transgene expression." Mol Cell Biol **26**(17): 6357-6371.

Yan, X., Z. Liu and Y. Chen (2009). "Regulation of TGF-beta signaling by Smad7." Acta Biochim Biophys Sin (Shanghai) **41**(4): 263-272.

Yang, P., Y. Wang, J. Chen, H. Li, L. Kang, Y. Zhang, S. Chen, B. Zhu and S. Gao (2011). "RCOR2 is a subunit of the LSD1 complex that regulates ESC property and substitutes for SOX2 in reprogramming somatic cells to pluripotency." Stem Cells **29**(5): 791-801.

Yang, X. J. and S. Gregoire (2005). "Class II histone deacetylases: from sequence

to function, regulation, and clinical implication." Mol Cell Biol **25**(8): 2873-2884.

Yang, X. J. and E. Seto (2008). "The Rpd3/Hda1 family of lysine deacetylases: from bacteria and yeast to mice and men." Nat Rev Mol Cell Biol **9**(3): 206-218.

Yeom, Y. I., G. Fuhrmann, C. E. Ovitt, A. Brehm, K. Ohbo, M. Gross, K. Hubner and H. R. Scholer (1996). "Germline regulatory element of Oct-4 specific for the totipotent cycle of embryonal cells." Development **122**(3): 881-894.

Ying, Y., X. M. Liu, A. Marble, K. A. Lawson and G. Q. Zhao (2000). "Requirement of Bmp8b for the generation of primordial germ cells in the mouse." Mol Endocrinol **14**(7): 1053-1063.

Ying, Y. and G. Q. Zhao (2001). "Cooperation of endoderm-derived BMP2 and extraembryonic ectoderm-derived BMP4 in primordial germ cell generation in the mouse." Dev Biol **232**(2): 484-492.

Yuri, S., S. Fujimura, K. Nimura, N. Takeda, Y. Toyooka, Y. I. Fujimura, H. Aburatani, K. Ura, H. Koseki and H. Niwa (2009). "Sall4 is essential for stabilization, but not for pluripotency, of embryonic stem cells by repressing aberrant trophectoderm gene expression." Stem cells **27**(4): 796-805.

Zhang, C. and M. W. Klymkowsky (2007). "The Sox axis, Nodal signaling, and germ layer specification." Differentiation **75**(6): 536-545.

Zhang, Y., Y. Wang, Q. Zuo, D. Li, W. Zhang, C. Lian, B. Tang, T. Xiao, M. Wang, K. Wang and B. Li (2016). "Effects of the Transforming Growth Factor Beta Signaling Pathway on the Differentiation of Chicken Embryonic Stem Cells into Male Germ Cells." Cell Reprogram **18**(6): 401-410.

Zhang, Y., Q. Zuo, Z. Liu, D. Li, B. Tang, T. R. Xiao, C. Lian, Y. Wang, K. Jin, Y. Wang, W. Zhang and B. Li (2015). "The Induction Effect of Am80 and TSA on

ESC Differentiation via Regulation of Stra8 in Chicken." PLoS One **10**(11): e0140262.

Zhang, Z., A. K. Elsayed, Q. Shi, Y. Zhang, Q. Zuo, D. Li, C. Lian, B. Tang, T. Xiao, Q. Xu, G. Chang, G. Chen, L. Zhang, K. Wang, Y. Wang, K. Jin, Y. Wang, J. Song, H. Cui and B. Li (2015). "Crucial genes and pathways in chicken germ stem cell differentiation." J Biol Chem **290**(21): 13605-13621.

SUMMARY IN KOREAN

원시 생식 세포는 유전 정보를 다음 세대로 전달해주는 유일한 세포인 정자 및 난자의 전구체이다. 다른 세포와는 구별되는 이 특성은 생식 세포 특이적 유전자 발현에 의해 조절된다. 원시 생식 세포는 전사 인자, RNA 결합 단백질 그리고 특이적 후생 유전학적 변형에 의해 조절된다.

*Nanog*는 종간에 그 단백질 서열이 유사하며 배아 발달 단계의 초기 배아 형성 과정의 핵심 인자로 잘 알려져 있다. 이 유전자는 또한 원시 생식 세포의 발달 과정에서, 전능성 세포에서부터 생식 선까지의 세포에서 발현하며, 이는 이 유전자가 생식 세포 발달 과정에서 줄기능과 생식능을 포함한 생식 세포의 특성을 전반적으로 유지함에 있어 중요한 기능을 한다. 실제로, *Nanog*는 다른 전사 인자들과 함께 신호 기작을 이용하여 원시 생식 세포를 조절한다. 뿐만 아니라, 이 유전자 만으로도 쥐의 배반엽 유사 줄기 세포에서 원시 생식 세포 유사 세포를 유도하는 것이 가능하다.

닭에서는 *Nanog*가 초기 발달 과정부터 관찰되며, 이후, 닭의 발달 단계인 HH3 부터는 이 유전자의 발현이 원시 생식 세포에 국한되는 양상을 확인하였다. 이 결과로부터 닭에서의 *Nanog*가 원시 생식 세포의 특성을 조절하는 핵심 인자일 것이라 추측할 수 있다.

최근 연구에서는 유전체에서의 5mC 의 소실, DNA 메틸레이션, 히스톤 및 크로마틴의 변형 등과 관련된 후생 유전학적 조절이 또한 원시 생식 세포에서 핵심적 기능을 함을 밝혀내고 있다. 그러나, 히스톤과 크로마틴의 변형 및 닭에서의 후생 유전학적 패턴에 대한 연구는 매우 적다.

이전의 연구에서, 생식 세포의 특성 결정부터 분화가 일어나는 동안의 메틸레이션과 아세틸레이션은 쥐의 생식 세포 특이적 유전자의 발현을 활성화시키고 체세포 유전자를 억제하며, 닭에서도 또한 후생유전학적으로 조절됨이 밝혀졌다. 그러나 포유류와는 다르게 닭에서는 H3K27me3 의 전반적인 레벨이 감소하고, 반면에 H3K9me3 의 레벨은 증가하였으며, 아세틸레이션의 경우에는 원시 생식 세포에서의 연구가 발현되지 않았다.

본 연구에서는 *Nanog* 의 전사적 및 후생 유전학적 조절 기작에 대해 알아보았다. 히스톤 탈아세틸화 효소인 HDAC 이 원시 생식 세포에서의 이 유전자를 조절하며, 또한, 이 유전자의 상부의 프로모터 부위에서의 메틸레이션이 감소되며 이 프로모터 부위에 결합하는 전사 인자들을 선별하였다. 이 연구의 결과는 닭에서의 원시 생식 세포 발달이 특이적 후생 유전적 조절을 받는다는 사실을 입증하였다.

추가적으로, 우리는 *Nanog* 가 직접적으로 VASA 를 조절함을 유전자의 과발현 및 낙다운, 면역 세포 화학 반응, 루시퍼레이즈 분석, 전사인자 분석 및 웨스턴 블롯을 통해 검증하였다. 이 결과는 조류의 원시 생식 세포가 포유류와는 다른 분자 조절 기작을 가질 수 있음을 의미한다.

결론적으로, 닭의 원시 생식 세포는 특이적인 *Nanog* 전사적 및 후생 유전학적 조절 기작을 가지며 이를 통해 생식능 획득 및 조절의 생물학적 이해를 풀고자 하였다. 본 연구는 또한, 발달 생물학 및 종 특이성의 연구에 대한 중요한 모델로서 적용 가능하다.